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The Chemistry of Flavonoids in Model Beverages and Human Milk

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THE CHEMISTRY OF FLAVONOIDS IN MODEL BEVERAGES AND HUMAN MILK

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Submitted to the Faculty

of

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by

Brian Jay Song

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of

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To my parents,

Ki Hwan and Hyon Sik Song,

who made my growth and prosperity possible.

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LIST OF ABBREVIATIONS

2,6-Di-tert-butyl-4-methylphenol (BHT)
Activation energy (E_a)
Acylated moiety (Acyl)
Analysis of variance (ANOVA)
Ascorbic acid (AA)
Bilitranslocase (BT)
Catechin (C)
Catechol-O-methyltransferases (COMT)
Cytosolic β –glucosidase (CβG)
Deoxyribonucleic acid (DNA)
Diode array detector (DAD)
Electrospray ionization in negative ion mode (ESI-)
Epicatechin (EC)
Epicatechin gallate (ECG)
Epigallocatechin (EGC)
Epigallocatechin gallate (EGCG)
Glucose transporter 2 (GLUT 2)
Glycoside (Gly)
Green tea extract (GTE)
High temperature short time (HTST)
Interquartile range (IQR)
Lactase phloridzin hydrolaze (LPH)
Limits of detection (LOD)
Liquid chromatography (LC)
Monocarboxilic acid transporter (MCT)
Mass spectroscopy (MS)
Multidrug resistance protein 2 (MRP2)
Multiple reaction monitoring modes (MRM)
Na_2-ethylenediaminetetraacetic acid (EDTA)
Non-fat dry milk (NFDM)
P-glycoprotein (PgP)
Quarter strength milk (QSM)
Phenolsulfosyltransferases (SULT)
Purple sweet potato (PSP)
Reactive oxygen species (ROS)

Ready to drink (RTD)
Single ion responses (SIRs)
Single strength milk (SSM)
Sodium-dependent glucose transporter (SGLT1)
Standard deviation (STDEV)
Standard error of mean (SEM)
Theasinesin (THSN)
Time of flight (TOF)
UDP-glucuronosyltransferases (UGT)
Vitamin enriched water (VEW)
Zero protein control (ZPC)

ABSTRACT

Song, Brian J. Ph.D., Purdue University, December 2013. The Chemistry of Flavonoids in Model Beverages and Human Milk. Major Professor: Mario G. Ferruzzi.

Interest in the chemistry and stability of flavonoids in foods has been generated by countless epidemiological, *in vitro*, and *in vivo* studies that suggest a variety of potential health promoting effects of diets rich in flavonoids. Flavonoids have been shown to interact with multiple components in beverages including macromolecules, minerals, certain vitamins, and other flavonoids. Furthermore, environmental conditions including light exposure, elevated temperatures, and relative humidity have been shown to modify flavonoid stability. However, specific mechanisms, relative kinetics of degradation reactions and the impact of macromolecules in model beverages and biological fluids on flavonoid stability remain largely unknown.

Recently, interest in the application of natural pigments to replace synthetic dyes in beverages has grown. However, there is limited information concerning the impact of prominent beverage ingredients, photo and thermal stress, and the potential mechanisms involved in anthocyanin degradation. **Chapter 2** of this dissertation investigates the stability of anthocyanin rich grape and purple sweet potato (PSP) extracts to photo and thermal stresses in ready-to-drink beverage models including hot-fill beverages with varying concentrations of ascorbic acid, a preserved beverage, and a vitamin enriched

water beverage. Thermal and photo stress were induced at 40, 60, and 80 ^{o}C and 250, 500, and 750 W/m^{2}, respectively. Increasing concentration of ascorbic acid resulted in more rapid degradation of anthocyanins through thermal stress, but had a protective effect through photo stress. Additionally, PSP was significantly less stable than grape extract in the vitamin enriched water model beverage through photo stress. Furthermore, a potential degradation mechanism involving the formation of monoacylated peonidins from diacylated peonidins under photo, but not thermal, stress was identified.

The transfer of dietary flavonoids to human milk and the potential exposure to nursing infants quickly became of interest. **Chapters 3 and 4** of this dissertation focuses on the presence of several flavonoids in human milk samples. In our initial screening, samples were collected from 17 women who delivered healthy term babies (37 wk of gestation) at 1, 4, and 13-wk postpartum intervals. Epicatechin (63.7–828.5 nmol/L), epicatechin gallate (55.7–645.6 nmol/L), epigallocatechin gallate (215.1–2364.7 nmol/L), naringenin (64.1–722.0 nmol/L), kaempferol (7.8–71.4 nmol/L),hesperetin (74.8–1603.1 nmol/L), and quercetin (32.5–108.6 nmol/L) were present in human milk samples with high inter/intraindividual variability. With the exception of kaempferol, the mean flavonoid content in human milk was not statistically different among lactation stages. In contrast, carotenoids in human milk significantly decreased from weeks 1 to 13 of lactation. In our follow up study, cohorts from the USA, China, and Mexico each comprised of 20 women in each country. Milk samples were collected at 2, 4, 13, and 26-wk postpartum intervals. Glucuronides of epicatechin, naringenin, kaempferol, hesperetin, and quercetin were the predominant metabolites detected. Despite all efforts,

anthocyanins were not detected in any of the 4 cohorts between the 2 studies and their relatively low bioavailability and presence in tissue is likely a contributing factor.

The presence of flavonoids in human milk suggested that select flavonoids maintained relative stability in protein rich fluid in order to avoid degradation between emptying through feeding. Previous studies indicated that polyphenols bind to proline rich segments, including those on caseins, and may have a stabilization effect. **Chapter 5** investigates the impact of protein rich milk on tea flavan-3-ol degradation during thermal treatment. Single strength milk (36.2 protein per L), quarter strength milk (9.0 g protein per L), and a zero protein control models were incubated with isolated epigallocatechin gallate and green tea extract at 62 or 37^{o}C for 180 minutes. Intact flavan-3-ols as well as auto-oxidation products including theasinesins (THSNs) and P-2 dimer concentrations were quantified by LC-MS. In general, greater polyphenol to protein ratios increased first order degradation rates, which consequently decreased the formation of THSNs and P-2 dimers. The presence of the galloyl and hydroxy moieties increased apparent affinity of flavan-3-ols to proteins, thereby stabilizing monomeric flavan-3-ols to processing conditions with increasing protein concentrations. In contrast, the absence of these moieties led to no observable interactions to proteins. A thorough understanding of protein-polyphenol interactions may provide further insight to their chemistry in protein rich beverages and biological fluids

CHAPTER 1. REVIEW OF LITERATURE

1.1 Introduction

Flavonoids, a class of phytochemicals, are nonnutritive plant secondary metabolites and are ubiquitous throughout the plant kingdom. Thus, they are commonly incorporated into the human diet as fruits, vegetables, and their derived beverages. In this dissertation, the stability, molecular interactions, and distribution to select tissues of various flavonoids are discussed. However, in order to justify and comprehend the described research, one must review the literature associated with this field of study. This chapter will discuss the molecular structures, biosynthesis, dietary sources, implications on human health, bioavailability, degradation mechanisms, and polyphenol-protein interactions. Finally, the research aims for the dissertation will be presented.

1.2 Flavonoid Molecular Structures

All flavonoids have a shared carbon backbone that consists of fifteen carbon atoms and one oxygen atom. Twelve carbon atoms are arranged as two separate six carbon aromatic rings (A- and B-rings) joined by one oxygen and three carbon atoms. Two carbons from the A ring are bound to a carbon and oxygen, resulting in a non-aromatic ring (C-ring) [**Figure 1.1**].[1-3] Flavonoids are characterized into one of many subclasses based on the oxidation state of the C-ring and substitution of the aromatic

rings. These subclasses include, but are not limited to, flavan-3-ols, anthocyanins, flavonols, flavanones, isoflavones, and flavones. However, this dissertation primarily concerns the first four subclasses of flavonoids mentioned above and will thus be the focus of the detailed review.

1.2.1 Flavan-3-ol Molecular Structure

The molecular structures of flavan-3-ols, also referred to as flavanols, are characterized by the lack of a double bond on the C-ring, and the presence of either a 3-hydroxy or 3-O-gallate moiety.[1] Monomeric flavan-3-ols exist in foods and beverages as catechins and their epimers, epicatechins. Both catechin (C) and epicatechin (EC) have hydroxyl moieties on the 7, 5, 4', and 5' positions on the A and B rings [**Figure 1.2**].[4] However, different substituents may be present on either the 3' or 3 positions. For instance, 3-O-gallate and 3'-hydroxy moieties are present on epicatechin gallate (ECG) and epigallocatechin (EGC) molecules, respectively. The presence of these moieties are not mutually exclusive, as observed in epigallocatechin gallate (EGCG). Unlike most other flavonoids, catechins and epicatechins are generally present as aglycones in plants.

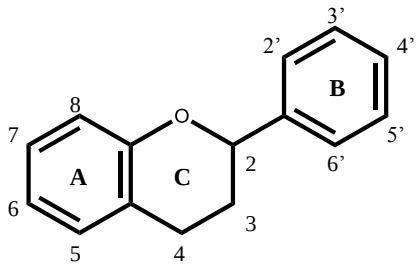
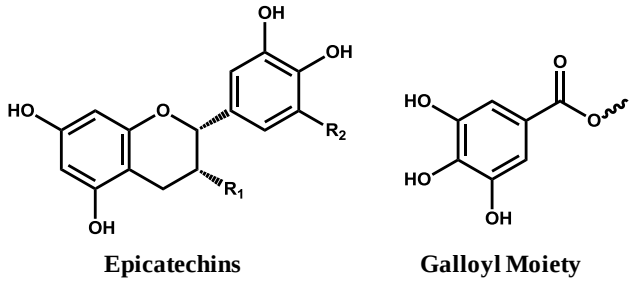


Figure 1.1 Basic Structure of Flavonoids Consisting of A, B, and C Rings.

Furthermore, it should be noted that polymeric flavan-3-ols, including various proanthocyanidins, are abundant in many plants. They are structurally analogous to their monomeric counterparts, but are bound by the 4, 6, or 8 positions and have been thoroughly reviewed.[4-6] However, the chemistry of polymeric flavan-3-ols is not the focus of this dissertation, thus the structures will not be discussed any further.

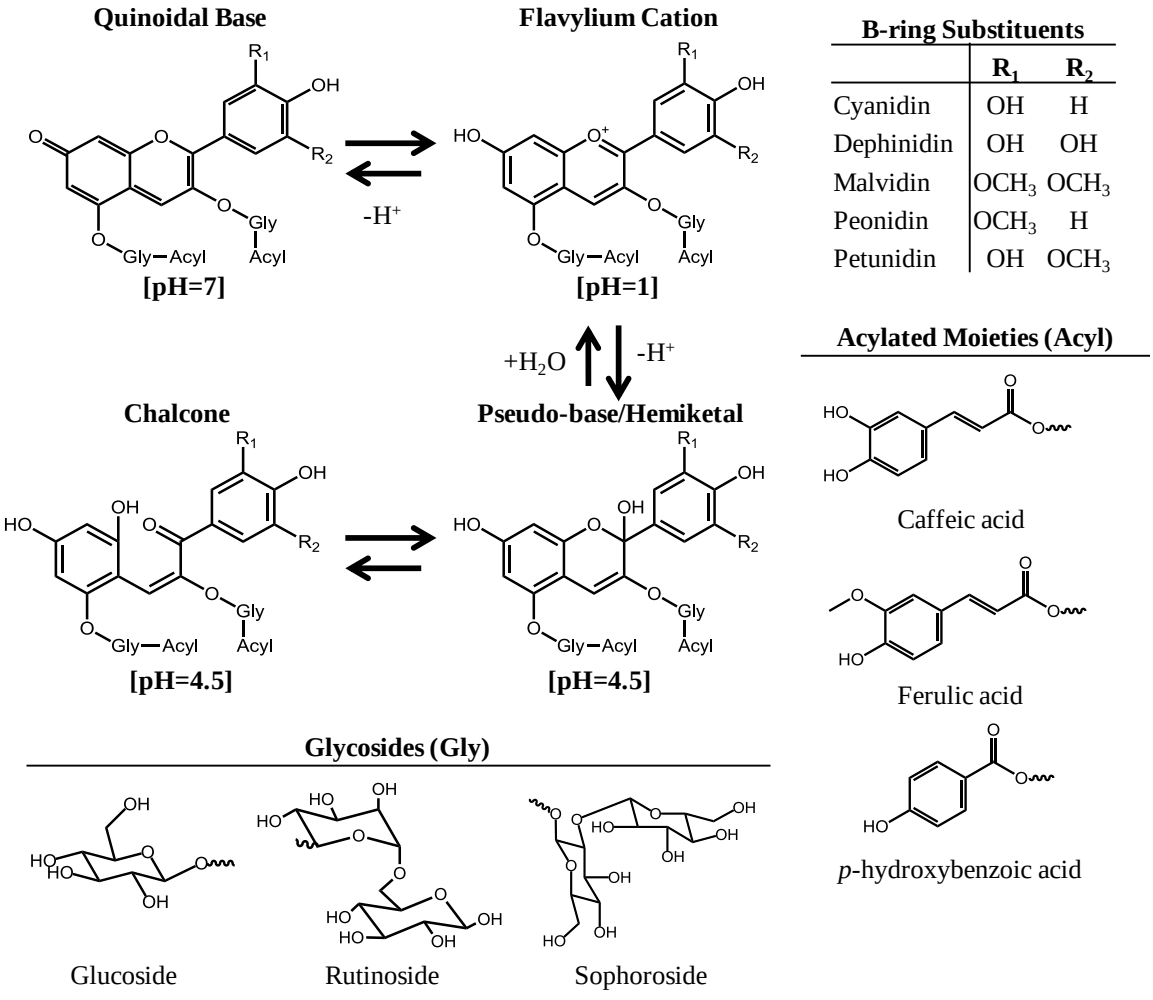
1.2.2 Anthocyanidin and Anthocyanin Molecular Structure

Anthocyanidins are under constant equilibrium between multiple different molecular configurations. However, the pH of the environment heavily favors certain configurations over others. In literature, the flavylium cation isomer is typically illustrated to highlight anthocyanidin structure and is the predominant structure at pH 1. However the quinonoidal base, carbinol pseudo-base (hemiketal) and chalcone forms are the major species at pH 7 and 4.5, respectively [**Figure 1.3**].[7] In general, malvidin, delphinidin, peonidin, petunidin, cyanidin, and pelargronidin are the predominant anthocyanidins associated with human consumption. All anthocyanidins are substituted with 4', 3, 5, and 7-hydroxy groups on their flavonoid skeleton. However, each anothcyanidin may have methoxy, hydroxy, or hydrogen substituents attached to their 3' and 5' positions.[1-3] Unlike flavan-3-ols, anthocyanidins are almost exclusively found as their analogous glycosides, referred to as anthocyanins. Predominant glycosides include, but are not limited to mono- or di-, glucoside, galactoside, sophoroside, rutinoside, and sambubioside on the 3- or 5- position.[8] Furthermore, anthocyanins may also be acylated with the presence of moieties including phenolic acids such as ferulic, p-hydroxybenzoic



Compound	R1	R2
Epicatechin	OH	H
Epigallocatechin	OH	OH
Epicatechin gallate	Galloyl	H
Epigallocatechin gallate	Galloyl	OH

Figure 1.2 Structures of Epicatechins.



B-ring Substituents	R_1	R_2
Cyanidin	OH	H
Dephinidin	OH	OH
Malvidin	OCH_3	OCH_3
Peonidin	OCH_3	H
Petunidin	OH	OCH_3

Figure 1.3 Structures of Anthocyanidins, Glycosides (Gly), and Acylated Moieties (Acyl).

and caffeic acids attached to their individual glycoside moieties.[9] Thus, many different configurations of a single anthocyanidin exist in nature.

1.2.3 Flavonol and Flavanone Molecular Structures

Flavonols and flavanones both contain 4-oxo substituents on their C-rings, but a double bond is present across the 2 and 3 positions on flavonols and not flavanones [**Figure 1.4**].[1-3] Quercetin and kaempferol are common dietary flavonols and may be present in plants as aglycones or glycosides.[2] Typical flavonol glycosides include glucosides, galactosides, and rhamnosides.[10] Naringenin and hesperetin are common dietary flavanones and are primarily present in plants as glucosides and rutinosides.[11]

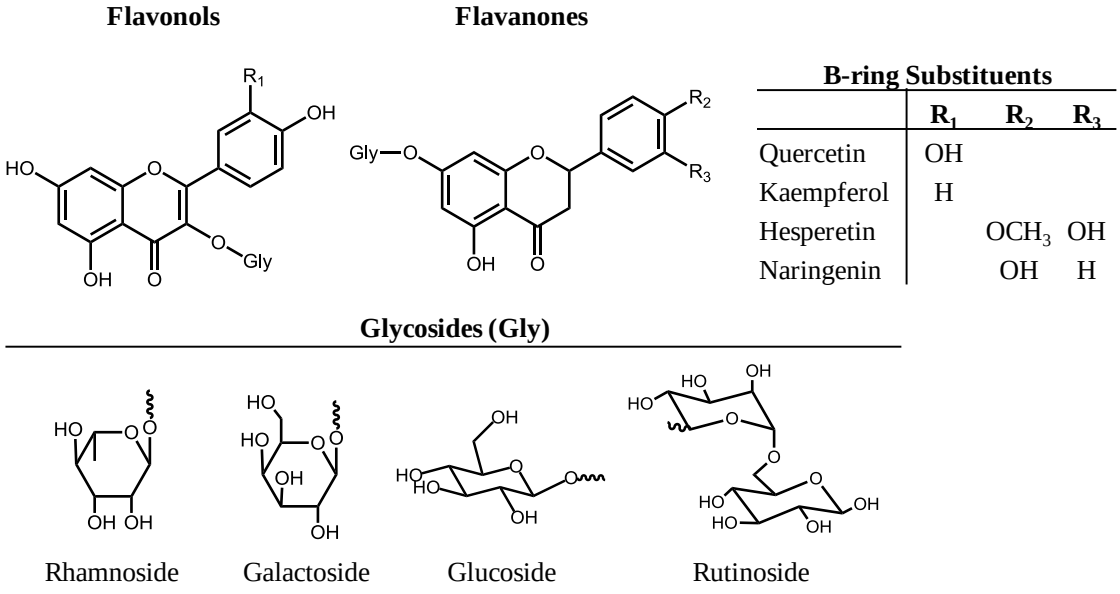


Figure 1.4 Structures of Flavonols, Flavanones, and Their Glycosides.

1.3 Biosynthesis of Flavonoids

All flavonoids are biosynthesized from the common precursors malonyl-CoA acid and *p*-coumaroyl-CoA. Acetyl-CoA is a substrate for acetyl-CoA carboxylase and produces malonyl-CoA [**Figure 1.5**].[12] The production of *p*-coumaroyl-CoA, relies on the formation of chorismate from the shikimate pathway. Through a series of enzymes, chorismate can produce phenylalanine.[13] Phenylalanine is converted to cinnamic acid by phenylalanine ammonia-lyase. Further enzymatic reactions lead into various secondary metabolite biosynthetic pathways. For instance, cinnamic acid is converted to *p*-coumaric acid and *p*-coumaroyl-CoA through sequential reactions by cinnamate 4-hydroxylase and *p*-coumarate:CoA ligase, respectively.[12] However, because this dissertation primarily focuses on flavonoids, only the biosynthesis of these compounds will be described further.

Three molecules of malonyl-CoA and one molecule of *p*-coumaroyl-CoA are converted into a single molecule of naringenin-chalcone by chalcone synthase. Chalcone isomerase may convert naringenin-chalcone to the flavanone naringenin in the typical 15 carbon arrangement of flavonoids [**Figure 1.1**]. Naringenin is a substrate for multiple enzymes, including flavanone 3-hydroxylase which results in the formation of dihydrokaempferol. Further reaction with flavonol 3'-hydroxylase converts dihydrokaempferol to dihydroquercetin.[12] Both of these dihydroflavonols are substrates for flavonol synthase and will produce kaempferol and quercetin. Glucosides are attached to flavanones and flavonols by various UDP-glucose:flavanone-7-O-glucosyl-transferase and UDP-glucose:flavanol-7-O-glucosyl-transferase, respectively.[14-15] Dihydroflavonols may also be converted to leucoanthocyanidin by dihydroflavonol 4-

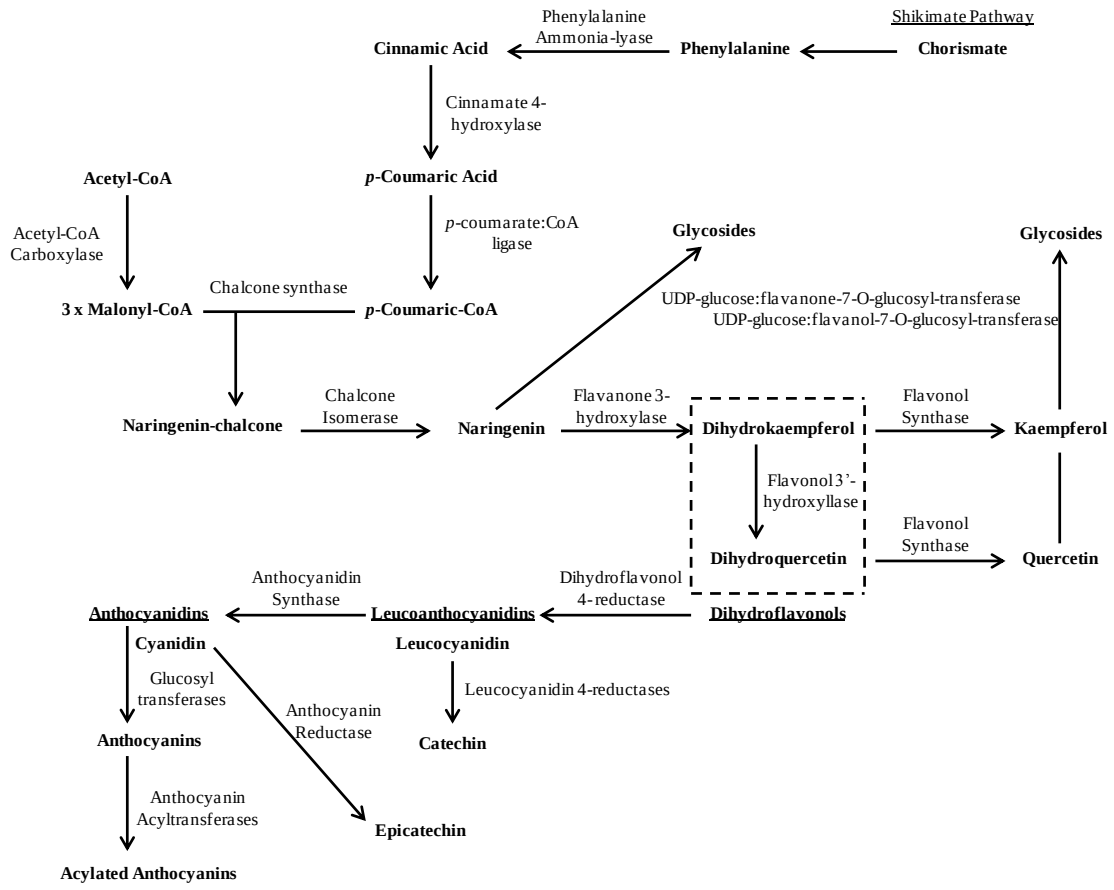


Figure 1.5 Biosynthetic Pathway of Flavonoids.

Table 1.1 Estimates and Composition of Dietary Flavonoids.

	Chun et al.[16]	*McCullough et al.[17]	Zamora-Ros et al.[18]	*Mink et al.[19]
Cohort	U.S. adults	U.S adults	EPIC-Spain	Iowa Women's Health Study
Years	1999-2002	1999-2006	1992-1996	1986-1997
Total Flavonoids (mg/day)	186.6	201.9	313.26	238.9
Flavonols (%)	6.8	6.4	6.0	3.7
Flavones (%)	0.8	0.5	1.1	0.2
Flavanones (%)	7.6	8.6	16.1	16.9
Flavan-3-ols (%)	83.5			
Monomers (%)	NR	8.3	10.4	8.5
Polymers (%)	NR	65.4	60.4	73.3
Anthocyanidins (%)	1.6	4.9	6.0	0.0
Isoflavones (%)	0.6	0.18	0.1	0.1

***** indicates data corresponding to the middle quintile of flavonoid intake. NR indicates that the data was not reported in the study.

reductase. Leucoanthocyanidins are substrates for anthocyanidin synthase and will produce anthocyanidins.[20-21] Furthermore, leucocyanidin, a specific leucoanthocyanindin, may be converted to catechins by leucocyanidin 4-reductase. Similarly, cyanidin, a specific anthocyanidin, may be converted to epicatechins by anthocyanidin reductase.[12] Finally, anthocyanidins can be glycosylated or acylated to anthocyanidin by glucosyltransferases and anthocyanin acyltransferases, respectively.[20-21]

1.4 Common Dietary Sources of Flavonoids

Studies have estimated that the average flavonoid intake for adults is between 189.7 to 313.26 mg/day and is largely dependent on dietary habits.[16-19] The relative consumption of specific flavonoid classes of free living humans provide further insight to their dietary exposure. Flavan-3-ols generally comprise the largest portion of all consumed flavonoids. Roughly 60 - 70 % and 8 - 10% of all consumed flavanoids are comprised of polymeric and monomeric flavan-3-ols, respectively. Flavanones and flavonols comprise the second and third greatest portion of dietary flavonoids at roughly 7 - 17 % and 3 - 7 %, respectively. The remaining dietary flavonoids consist of anthocyanidins, flavones, and isoflavones **[Table 1.1]**.[16-19] As discussed earlier, flavonoids are ubiquitous throughout the plant kingdom, therefore dietary sources primarily include fruits, vegetables, and their derived beverages. Typically, groups of foods and beverages are associated with high concentrations of certain flavonoid classes. For example, flavan-3-ols, anthocyanins, and flavanones are predominantly present in teas, berries, and citrus fruits, respectively.[22] In this section, the common dietary sources of flavonoids relevant to this dissertation will be discussed.

1.4.1 Flavan-3-ols

The majority of dietary flavan-3-ols are comprised of monomeric and polymeric flavan-3-ols. Both are present in high concentrations in many foods and beverages including chocolate, various teas, and wine.[22] However, polymeric flavan-3-ols, with the exception of B-ring dimers, are not the focus of this dissertation, thus their common dietary sources will not be described here but have been discussed in other studies.[23-26] Furthermore, B-ring dimers are formed through the auto-oxidation of flavan-3-ols and are a result of food processing and not native to plant material. Thus, their structure, presence, and chemistry are described in detail in the flavonoid stability section of this literature review. Teas are the second most widely consumed beverage in the world and are typically rich in flavan-3-ols, specifically the epicatechins. Although EGCG is typically the predominant species, teas also contain a substantial concentration of EC, EGC, and ECG. Flavan-3-ol profiles of teas can greatly vary depending on growing region, environmental conditions, and cultivars.[27-28] Furthermore, the degree of "fermentation" determines the oxidation of the tea leaves and directly impacts the flavan-3-ol concentrations. Thus, green tea typically has the greater flavan-3-ol concentration than oolong and black teas **[Table 1.2]**.[29-31]

Teas are considered the most abundant source of multiple monomeric flavan-3-ol species. However, other foods and beverages are considered common dietary sources because of their high concentration of specific flavan-3-ols. For example, C and EC, are prominent in the phenolic profiles chocolate, wines, and fruits including grapes, apples, and strawberries.[26, 32-37] Other flavan-3-ol species like EGC and ECG may also present in these foods, but are less common and typically present in lower concentrations.

Table 1.2 Concentration of Epicatechins (μmol/g Dry Leaf) in Teas.

Green Tea (μmol/g dry leaf)	EC	EGC	ECG	EGCG	Total
Longjin[a]	18.9	19.3	46.5	141	226
Bi-luo-chun[a]	11.4	60.8	29.9	155	257
Yun-nan-lu-cha[a]	50.4	5.23	143	103	301
Xin-yang-mao-jing[a]	28.2	11.8	60.5	228	328
Lu-zhu-cha[a]	20.0	11.4	30.4	124	185
Nagasaki (Japan)[b]	42.5	163	37.8	168	411
Zhejiang (China)[b]	35.5	71.9	70.5	187	365
Darjeeling[c]	34.3	52.0	91.7	137	315
Bold Leaf (Thailand)[c]	46.2	23.3	121	56.2	247
First Grade Bancha (Japan)[c]	37.4	69.7	81.7	126	315
Green Sencha (Vietnam)[c]	37.2	70.1	75.0	109	291
Gyokuro (Japan)[c]	38.0	77.6	78.9	149	344
First Grade Gunpowder (China)[c]	33.7	46.8	66.4	93.8	241
Chun Mee Moon Palace (China)[c]	36.3	66.2	82.7	142	327
Oolong Tea (μmol/g dry leaf)					
Wu-yi-wu-long[a]	12.1	6.86	16.1	79.0	114
Shui-xian[a]	13.2	5.23	16.5	61.1	96.1
Guan-yi[a]	13.2	8.17	16.3	76.2	114
Fujian (China)[b]	42.7	156	33.3	127	359
Guangdong (China)[b]	23.5	58.4	30.4	92.5	205
Premium Formosa Choicest[c]	28.2	13.2	43.1	38.3	123
Formosa Fine Grade[c]	29.5	19.8	50.6	52.1	152
Formosa Amber[c]	31.0	39.1	46.2	64.9	181
Se Chung (China)[c]	30.2	31.7	42.1	58.2	162
Formosa Jade[c]	31.5	41.4	49.7	75.5	198
Black Tea (μmol/g dry leaf)					
Fu-zhou-hong-cha[a]	Trace	Trace	2.49	2.84	5.33
Li-zhi-hong-cha[a]	1.79	Trace	3.85	3.50	9.13
Liu-an-gu[a]	3.93	0.327	1.81	6.78	12.8
Darjeeling (India)[b]	16.8	37.8	42.6	94.9	192
Uva (Sri Lanka)[b]	7.68	23.8	25.8	34.0	91.2
Orthodox BOP Darjeeling[c]	31.5	17.3	116	126	291
Arya Estate Darjeeling[c]	29.8	8.84	106	77.5	222
Premium Blend Darjeeling[c]	31.0	16.0	97.3	87.8	232
Poobong Estate Darjeeling[c]	30.1	17.8	86.9	115	250

[a,b,c] Data was obtained from Chen et al., Shii et al., and Nielson et al., respectively.[29-31] Catechin, gallocatechin, and gallocatechin gallate were not represented in this table because of inconsistent reporting in literature.

Although flavan-3-ols are present in many other foods and beverages, only a finite number of examples are presented in this literature but have been discussed elsewhere [**Table 1.3**].[24, 26, 32-33]

1.4.2 Anthocyanins

Recent estimates suggested that the average anthocyanin daily intake in the United States is 12.5 mg/person. Out of these anthocyanidins, 44.7% were cyanidin 20.7% delphidin, 15.4% malvidin, 9.0% petunidin, 6.9% peonidin, and finally 3.3% pelargonidin. Monoglycosides and diglycosides acounted for 73.1% and 17.3%, respectively. Finally, 77.0% of anthocyanins were non-acylated, while the

Table 1.3 Concentration of Monomeric Flavan-3-ols in Chocolates, Fruits, and Wines.

Chocolates (mg/kg WW)	**C**	**EC**	**GC**	**EGC**	**ECG**
Cocoa powder[a]	579	1853			
Baking chocolate[a]	491	1142			
Dark chocolate[a]	164	337			
Milk chocolate[a]	43.3	99.3			
Fruits (mg/kg WW)					
Apricot[b]	49.5	60.6			
Raw broad bean[b]	128.3	225.1		140.3	
Sweet Cherry[b]	21.7	95.3			
Granny smith apple[c]	6.00	26.6			
Red delicious apple[c]	15.7	63.7			
Strawberry[c]	15.7	0.2	1.2	1.5	6.6
Cabernet SauvignonGrape*					
Seed[d]	3140	2710			14
Skin[d]	206	171			
Wines (mg/L)					
Red Wine[c]	17.8	11.4	4.2	2.8	
Rose Wine[c]	7.1	3.7	1.8	0.7	
White Wine[c]	1	0.6	0.1		

[a,b,c,d] Data was obtained from Miller et al., Arts et al., Pascual-Teresa et al., and Monagas et al., respectively.[26, 32, 34, 36] * Flavan-3-ol concentration units for grape seed and skin were published on an mg/kg dry weight.

remainder was various acylated derivatives.[38] Anthocyanins are the bright blue and red pigments found in many plants and their fruits. Thus, blue, red, and black colored berries and vegetables are typically considered rich dietary sources of anthocyanins. However, the predominance of specific anthocyanidins, glycosides, and acylated moieties greatly vary between potential dietary sources, their varieties, geographic region and season.[38-40] Multiple anthocyanins are typically present in a single dietary source, however only a few anthocyanins generally comprise the vast majority of dietary forms typically consumed. For example, the majority of anthocyanins in berries are predominantly nonacylated mono-glucosides, galactosides, arabinosides, rutinosides, sophorosides, or xylosides.[38, 41] However, the anthocyanin in highest concentration varies between each type of berry. For instance, the predominant anthocyanins in bilberry are glucosides, galactosides, and arabinosides of either cyanidin or delphinidin.[42-43] Black currants are rich in both cyanidin and delphinidin rutinosides, but not glucosides or galactosides.[8, 43] Furthermore, blueberries, grapes, and red wine are typically reported to be particularly rich sources of malvidin 3-O-glucoside.[8, 44-46]

As previously discussed, estimates indicate that 23.0% of dietary anthocyanins are acylated.[38] In general, acylated anthocyanins are present at low and high concentrations in fruits and vegetables, respectively.[38] Furthermore, the food industry has recently been interested in the use of anthocyanin rich extracts, specifically those composed of high concentrations of acylated anthocyanins, for a natural alternative to synthetic food dyes.[39] Acylated anthocyanins have been associated with greater color stability then their non-acylated counterparts, in part because of their ability to participate in intramolecular copigmentation interactions.[47-48] Thus, food companies interested in natural pigments

have been focused on acylated anthocyanin rich extract including purple sweet potato, red cabbage, and red radish.[9, 49-50] Thus, the dietary intake of acylated anthocyanins may increase in the near future, particularly among consumers of ready to drink beverages. Purple sweet potatoes are particularly rich in peonidin and cyanidin 3-sophoroside-5-glucosides mono- or diacylated with a combination of caffeic, ferulic, and *p*-hydroxybenziuc acids.[9] Similarly, red cabbage is rich in cyanidin 3-O-diglucoside-5-O-glucoside mono- or diaclyated with caffeic, p-coumaric, ferulic, and sinapic acids.[49]

1.4.3 Flavonols

Recent estimates indicated that the average daily flavonol intake in the United States is 12.9 mg/person.[16] However, health workers in the United States were reported to have average daily flavonol intakes ranging from 19.9 to 23.4 mg/person, depending on gender and age.[51] Approximately 75 and 21% of consumed flavonols consisted of a quercetins and kaempferols, respectively.[51] Thus, the vast majority of dietary flavonols consist of either quercetin or kaempferol aglycones and therefore are typically the focus of many studies focusing on the health effects of flavonols. Although quercetin is considered almost ubiquitous in plants, tremendous variation exists between plant species and food or beverage products. For instance, onions and apples contribute to a large portion of dietary quercetin in the United States, but not in Italy, where wine is considered a major dietary source of quercetin.[52] Typically, onions, apples, berries, and their derived foods and beverages are rich with quercetin aglycone and glycosides including but not limited to mono/diglucosides, rutinoside, galactosides, arabinosides, rhamnoside, and xylosides.[53-57] In contrast, kaempferol is found in much lower

concentrations and in much fewer foods. Broccoli, kale, and some variety of tomatoes are reported to be major dietary sources of kaempferol.[58-61]

1.4.4 Flavanones

In general, the vast majority of dietary flavanones are either hesperetins or naringenins. In the European Prospective Investigation into Cancer and Nutrition, roughly 65% and 33% of dietary flavanones were hesperetins and naringenins, respectively.[18] Unlike flavonols and flavan-3ols, flavanones are typically only found in high concentrations in citrus fruits, tomatoes, and their derived food products. [61-62] Thus, these foods are considered the only significant contributor to flavanone intake. Furthermore, hesperetin and naringenin are predominantly present as rutinosides, but are also present as glucosides to a much lesser extent.[63-64]

1.5 Flavonoids and Health

The overarching goal of flavonoid research is to facilitate the development of disease preventative dietary recommendations and the design and evaluation of foods designed for specific health benefits. Thus, in all flavonoid research, it is important to discuss the potential impact that flavonoid consumption may have on specific aspects of human health.

1.5.1 Oxidative Stress

As previously discussed, the chemical structure of flavonoids consists of aromatic rings, conjugated double bonds and typically multiple hydroxyl substituents.[1-3] This

unique structure enable flavonoids to react with reactive oxygen species (ROS) producing a stabilized flavonoid radical.[65] While direct antioxidant activity is perhaps the best known "activity" related to flavonoids, these dietary compounds may reduce oxidative stress through other mechanisms, including disruption of nitric oxide synthesis and xanthine oxidase, as well as stimulation of endogenous antioxidant systems.[66] Oxidative stress and the oxidation of DNA, proteins, and lipids has been associated with negative health outcomes.[67] For example, human clinical trials indicated that the consumption of tea significantly increases plasma antioxidant capacities, thus relieving systemic oxidative stress.[68] The ability of specific flavonoids and their metabolites to directly or indirectly mitigate oxidative stress and protect vital biological molecules, cells and tissues through multiple mechanisms would be consistent with prevention and progression of several chronic and degenerative diseases.[69]

1.5.2 Inflammatory Stress

Inflammatory stress is related to oxidative stress and the development of diseases included cancer, cardiovascular, and neurodegenerative diseases. Inflammatory response involves the release of arachidonic acid from cells by phospholipase A_2, which is then metabolized by cyclooxygenases and lipoxygenases to produce multiple compounds which may harm a variety of biological components.[66, 70] However, flavonoids are able to disrupt multiple enzymes in the cascade of reactions associated with the inflammatory response, and thus mitigating cellular damage. For instance, quercetin and rutin inhibit phospholipase A_2 and thus prevent the release of arachidonic acid.[71-72] Quercetin and kaempferol inhibit the transcription of cyclooxygenase-2 and prevent the production of

prostaglandins and thromboxanes.[70, 73] Finally, flavan-3-ols, quercetin, kaempferol, and morin inhibit multiple lipoxygenases.[74-75]

1.5.3 Cardiovascular Disease

As previously discussed, the presence of ROS may induce lipid peroxidation and promote inflammation that is linked to the development of cardiovascular disease and atherosclerosis.[67] Furthermore, studies have shown that select flavonoids including quercetin can inhibit the expression of inducible nitric oxide synthase and cyclooxygenase-2, minimizing oxidative damage.[70] Thus, flavonoid rich diets may be capable of reducing the relative risks of cardiovascular diseases. Several epidemiological studies suggested that high dietary consumption of flavonoids reduced the relative risk of cardiovascular diseases. For example, a cohort of 5133 men and women in Finland found a significant inverse relationship between flavonoid intake and coronary mortality in women after major cardiovascular risk factors were considered.[76] Other epidemiological studies differentiated the impact of various groups of flavonoids on the reduced risk of cardiovascular disease. In a cohort of postmenopausal women from Iowa, an significant inverse relationship was found between anthocyanidin and flavanone consumption and the relative risk of cardiovascular and coronary heart diseases, respectively.[19] Similarly, the relationship between flavonols and reduced risk of cardiovascular diseases has been investigated, but a small portion of studies indicated no significant difference in risk with flavonol intake.[77]

1.5.4 Cancer

ROS have the ability oxidize DNA, which may cause permanent damage and may contribute to the initiation of cancer.[67] The consumption of flavonoids has been associated with reduced systemic oxidative stress, oxidized DNA, and may have the promise of reducing the relative risk of select types of cancer.[67] Furthermore, *in vivo* and *in vitro* studies found that certain flavonoids may inhibit cytochrome P450 enzymes and promote the activity of antioxidant enzymes including glutathione reductase and glutathione peroxidase.[78] However, epidemiological studies have reported varying results on different types of cancers and populations.[79] For instance, in a cohort of 10,054 Finnish men and women, men with higher quercetin and myricetin intakes had lower relative risks to lung and prostate cancers, respectively.[80] A multiethnic cohort from Hawaii and Los Angeles suggested that high flavonol consumption, particularly kaempferol, was inversely related to relative pancreatic cancer risk.[81] Additionally, a greater reduction in relative pancreatic cancer risk was observed among smokers than non-smokers, likely because of the additional oxidative stress caused by smoking.[81] In contrast, another study suggested that flavonoid intake and relative lung cancer risk reduction was strongly inversely related in younger (<50) populations and non-smokers.[82]

1.5.5 Neurodegenerative Diseases

The nervous system, specifically the brain, is particularly sensitive to inflammation and oxidative damage. Systemic oxidative stress is likely to impact the brain because it consumes a large quantity of oxygen, contains low concentrations of enzymes that metabolize ROS, and membrane lipids contain high concentrations of

polyunsaturated fatty acids.[83] Oxidative damage to the brain has been associated with both age related cognitive decline as well as several neurodegenerative diseases including Alzheimer's disease and Parkinson's disease.[84] As previously discussed, flavonoids may inhibit nitric oxide production and cyclooxygenase-2, reducing oxidative stress and inflammation, both of which have been associated with neurodegenerative diseases.[85] Thus, the consumption of flavonoid rich foods and beverages may hold the potential to reduce the relative risk of neurodegenerative diseases. In contrast to flavonoid research related to cardiovascular disease and cancer, there is a lack of epidemiological studies that focus on the relative risk of neurodegenerative diseases and flavonoid intake. A relatively small scale epidemiological study in France has associated flavonoid intake with reduced risk of cognitive decline.[86] Furthermore, the Kame project suggested that the consumption of fruit and vegetables juices is inversely associated with Alzheimer's disease risk, potentially due to an increase flavonoid intake.[87] These epidemiological studies have been additionally bolstered by both preclinical and *in vitro* studies which suggests that select flavonoid rich foods including green tea, blueberries, grape and grape derived products (juice, wine and seed extract) as well as Ginkgo biloba may hold promise in modifying risk factors and neurodegenerative processes and associated diseases.[88]

1.6 Flavonoid Absorption, Metabolism, and Bioavailability

In order for dietary flavonoids to deliver their potential as health promoting and/or disease modifying properties, they must first be absorbed from the gut lumen into the vascular system. During this process and throughout circulation, flavonoids are subject

to multiple conjugation reactions associated with phase II metabolism. These processes contribute to the generation of specific biologically relevant flavonoid metabolite forms that can be monitored by plasma pharmacokinetics and totally urinary elimination. This section will review the absorption, metabolism, and bioavailability of flavonoids.

1.6.1 Flavonoid Absorption

Directly after consumption, flavonoids are exposed to gastric conditions in the stomach. With the exception of anthocyanins, flavonoids are generally not absorbed in the stomach. Pharmacokinetic studies have reported that anthocyanins appear in plasma rapidly after consumption and suggested that they may be absorbed in the stomach.[89-90] Furthermore, a study using an *in situ* gastric administration of anthocyanins to rats confirmed that anthocyanins are transported from the gastric lumen.[91] Passamonti et al. demonstrate that anthocyanins are a substrate for bilitranslocase in both hepatic and gastric tissues.[92-93] Thus, anthocyanin transport by bilitranslocase in the gastric epithelium is a plausible explanation for their rapid appearance in plasma after consumption.[94] However, it should be noted that pharmacokinetic curves suggest that the stomach is not the predominant site of anthocyanin absorption.

The vast majority of flavonoid absorption occurs in the small intestines. Flavonoids are transported from the intestinal lumen into enterocytes by multiple potential mechanisms [**Figure 1.6**]. It is widely accepted that flavonoid glucosides are substrates for sodium-dependent glucose transporter (SGLT1), glucose transporter 2 (GLUT 2), monocarboxilic acid transporter (MCT) and lactase phloridzin hydrolaze (LPH) on the brush border membrane.[95-97] LPH hydrolyze flavonoid glycosides and

release their analogous aglycones into the intestinal lumen, which may then enter enterocytes by passive diffusion. Certain dietary aglycones like the flavan-3-ols, specifically ECG, have been shown to be a substrate for MCT and may serve as a mechanism for flavan-3-ol absorption.[98] SGLT1 transports intact flavonoid glucosides into enterocytes where they are substrates for cytosolic β –glucosidase (CβG) and multidrug resistance protein 2 (MRP2).[95, 97] Similar to LPH, CβG hydrolyzes flavonoid glucosides to release their aglycone, which are then conjugated by multiple enzymes and transported to the circulatory system. MRP2 and P-glycoprotein (PgP) are responsible for efflux flavonoid glucosides, aglycones and select intestinal metabolites back to the intestinal lumen, where they become available to react with SGLT1 or LPH.[95-98]

Flavonoids that are not absorbed in either the stomach or the small intestines are eventually moved further through the GI tract and to the colon. It should be noted that flavonoid metabolites may be excreted in bile via enteroheptatic circulation and thus may be present in the colon. For instance, methylated delphinidins were detected in bile of rats after oral administration.[99] Although the colon may not effectively absorb flavonoids, microorganisms can metabolize the flavonoid backbone by cleaving the heterocyclic oxygen containing ring to produce multiple products.[100] A study illustrated that colonic microflora convert flavonols to 3-hydroxyphenylacetic and homovanillic acids prior to colonic absorption and circulation.[101] Furthermore, other studies demonstrated that 3,4-dihydrozyphenylacetic, 3-phenylpropionic, 3-(3-hydroxyphenyl)-propionic, and protocatechuic acids are formed by quercetin and naringenin when exposed to microorganisms in a fecal slurry.[102]

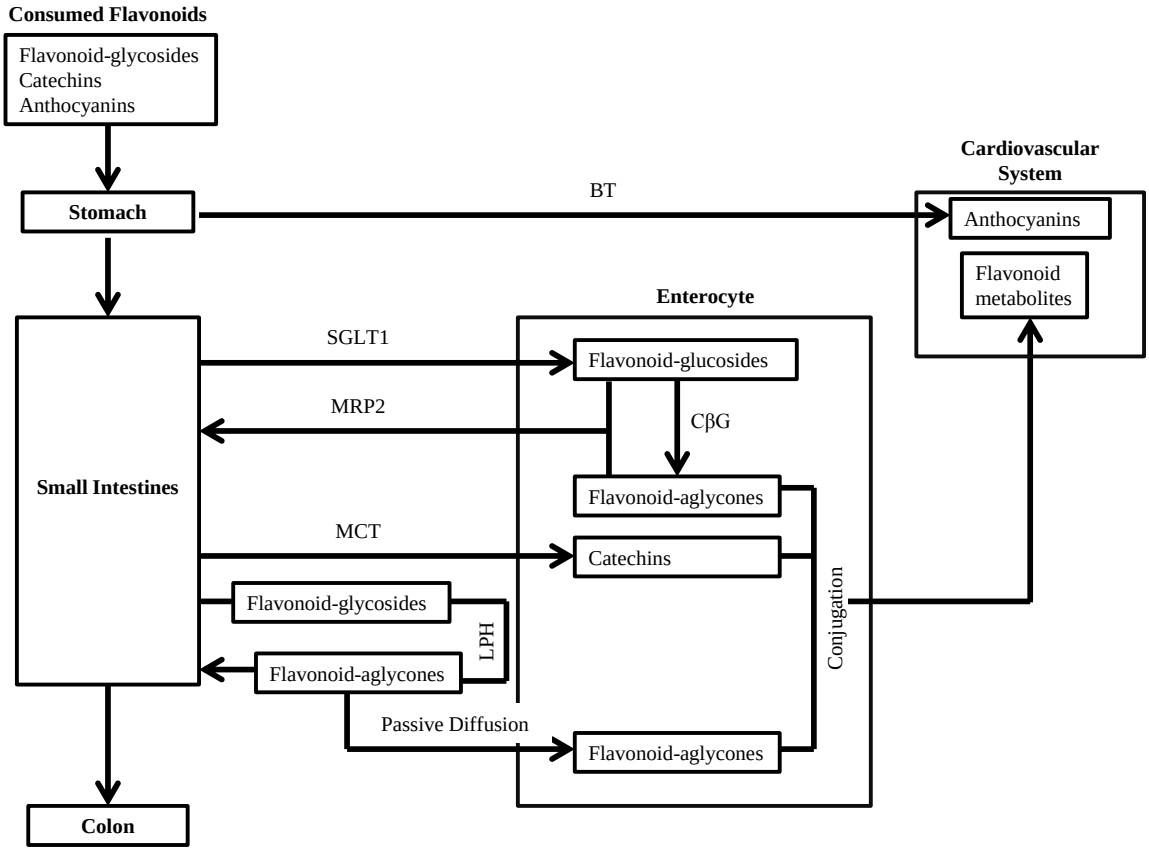


Figure 1.6 Absorption of Flavonoid Glycosides, Catechins, and Anthocyanins.
Enzymes are abbreviated as follows: bilitranslocase (BT), sodium-dependent glucose transporter (SGLT1), multidrug resistance protein 2 (MRP2), cyotsolic β –glucosidase (CβG), monocarboxylate transporter (MCT), and lactase phloridzin hydrolaze (LPH).

1.6.2 Flavonoid Metabolism

The majority of absorbed flavonoids are conjugated through phase II xenobiotic metabolism during circulation.[100] However, studies have suggested that unconjugated flavonoids are present in plasma during first pass circulation. For instance, anthocyanins that were absorbed in the stomach of rats were detected in plasma.[91] Furthermore, both unconjugated and conjugated green tea flavan-3-ols were present in the human plasma after their consumption.[103] Phase II xenobiotic metabolism occurs extensively in small intestinal enterocytes, liver, and kidney tissues.[104] Flavonoids are exposed to one or more metabolic reactions including glucuronidation, methylation, and sulfunation throughout absorption and circulation.[104] UDP-glucuronosyltransferases (UGT), catechol-O-methyltransferases (COMT), and phenolsulfosyltransferases (SULT) enzymes are responsible for these conjugation mechanisms.[95, 100, 104-105]

Subclasses of flavonoids tend to be metabolized similarly and favor certain conjugations. For instance, peonidin monoglucuronide and cyanidin 3-O-glucoside monoglucuronide were both identified in human urine after consumption of elderberry extract.[106] Furthermore, conjugations may occur at various positions, resulting in isomeric metabolites. Three different monoglucuronides and a sulfunated metabolite of perlargonidin were detected in human urine after strawberry consumption.[107] Flavonols, like quercetin, have been detected in plasma conjugated as glucuronides, methyl -glucuronides, and sulfates.[108] Similarly, hesperetin was found in human plasma predominately as glucuronide conjugates and sulfoglucuronide conjugates to a much lesser extent.[109] Green tea flavan-3-ols are conjugated through glucuronidation, sulfonation, and methylation.[103, 110-111]

1.6.3 Flavonoid Bioavailability

The diversity of flavonoid structures, absorption, and exposure to xenobiotic metabolism mechanisms cause great variability in their bioavailability. Furthermore, it is generally accepted that inter-individual variability heavily impacts the bioavailability of flavonoids. Flavonoid bioavailability is typically estimated by quantifying flavonoids and their metabolites in plasma or urine over a period of time. Because of the nature of these biological fluids, their use in bioavailability assessment has different strengths and weaknesses.[112] For instance, plasma pharmacokinetics will yield data more relevant to flavonoid absorption and metabolite circulation over a period of time, but is unable to determine total flavonoid absorption. In contrast, urinary elimination data yields more accurate data concerning total flavonoid absorption, but not the absorption as a function of time and cannot estimate the elimination of flavonoids in bile. Thus, many studies have focused on both plasma pharmacokinetics and total urinary elimination to obtain a thorough understanding of flavonoid bioavailability.

Studies have examined the pharmacokinetics and urinary elimination of flavonoids in humans and animal after being fed flavonoid rich foods/beverages or isolated flavonoids. Anthocyanins are generally accepted to have an extremely low bioavailability. For instance, urinary elimination of malvidin 3-O-glucoside six hours after consumption of dealcoholized red wine and red grape juice were 0.016% and 0.019% of their intake and maximum plasma concentrations reached 0.001 and 0.003 μM, respectively.[113] Other studies have focused on a variety of anthocyanins including, delphinidin and cyanidin glucosides and rutinosides which were present at 0.007 to 0.133% of consumed anthocyanins in urine after consumption of black currant juice.[114] Studies

have focused on the bioavailability of flavonols, specifically quercetin, after the consumption of foods/beverages and isolated quercetin. Maximum plasma concentrations were 0.34, 0.64, 2.12, and 2.31 µg/mL after ingestion of isolated rutin (200 mg quercetin equivalence), buckwheat tea (200 mg quercetin equivalence), isolated quercetin 4'-glucoside (100 mg quercetin equivalence), and onions (100 mg quercetin equivalence), respectively.[115] Urinary excretion coincided with the maximum plasma concentration data and were 0.9, 1.0, 4.5, and 6.4% of quercetin intake, respectively.[115] The bioavailability of flavanones, primarily hesperetin and naringenin, were investigated after the consumption of citrus juices or isolated compounds. Urinary excretion ranged from 4.1 to 6.4 and 7.1 to 7.8 % of hesperetin and naringenin intake after consumption of orange juice, respectively.[109] Similarly, the maximum plasma concentrations and total urinary excretion of hesperetin and naringenin were 2.7 and 7.4 µM, and 3.3 and 5.8 % after consumption of flavanone isolates, respectively.[116] Finally, the bioavailability of monomeric flavan-3-ols has commonly been investigated through the consumption of apples, cocoa, grape seed and tea extracts. After the consumption of green tea extract, urinary excretion accounted for a trace, 15.6 %, and 8.9 %, intake of EGCG, EGC + methylated EGC, and EC, respectively. Furthermore, maximum plasma concentrations reached 0.17, 5.78, and 0.43 µM, respectively.[117]

1.6.4 Flavonoid Tissue Distribution

After flavonoids are absorbed and metabolized, they may be distributed throughout the body and into various tissues via the circulatory system. Their ability to reach target tissues directly impacts their interaction with biological mechanisms which

may reduce the relative risk of diseases. However, in order to reach these tissues, flavonoid metabolites must be transported through blood-tissues barriers. For instance, tea flavan-3-ols were found to be distributed in the esophagus, prostate, spleen, bladder, lung, kidney, liver, and heart in rats.[118] Anthocyanins were found in stomachs, kidney, liver, bladder, prostate, testes, heart, adipose tissue and brain in rats.[119-120] Quercetin were distributed into the liver, kidney, muscle, heart, lung, brain, testes, spleen, thymus, bone, and fat in rats.[121] Furthermore, quercetin was found in the liver, kidney, lung, spleen, and brains of pigs.[121-122] Franke et al. investigated the content of isoflavones in human milk after soy consumption.[123-124] However, there is very limited information concerning the distribution of other flavonoids including anthocyanins, flavan-3-ols, flavonols, and flavanones in human milk. Since a mother's milk is the primary source for an infant's nutrition, research concerning the flavonoid content in human milk merits investigation.

1.7 Flavonoid Stability

In order for flavonoids to be available for absorption and potentially impart their disease preventative properties, they must withstand conditions commonly associated with food and beverage production, storage, and digestion. However, flavonoids are prone to multiple degradation reactions induced by oxygen, neutral or basic pH, elevated temperatures, and light exposure. Furthermore, simultaneous exposure to a combination of these conditions has a synergistic effect on flavonoid degradation. A multitude of studies have focused on the behavior of various flavonoids throughout these conditions and are reviewed in this section.

1.7.1 Oxygen and Oxidation

As previously discussed, flavonoids are antioxidants and readily react with ROS. Therefore, the presence of molecular oxygen or ROS during processing or storage will accelerate their degradation. For instance, the aeration of juice samples was determined to significantly accelerate the deterioration of color in anthocyanin rich juices.[125] Furthermore, anthocyanins showed greater instability in containers where the headspace was comprised of air rather than to nitrogen or vacuum headspaces.[126] Similarly, exposure of quercetin to atmospheric oxygen significantly contributed to the decomposition and formation of oxidation products.[127] The sensitivity of flavonoids to oxygen is not limited to atmospheric oxygen, but also includes dissolved oxygen in aqueous systems. For instance, EGCG degradation rates significantly increased with increasing concentrations of dissolved oxygen.[128]

The initial degradation of flavonoids through oxidation tends to follow a common mechanism. First, a radical semiquinone structure is formed and is stabilized via multiple resonance structures available to the flavonoid.[129] Further oxidation will result in the formation of a stabilized quinone structure **[Figure 1.7]**.[130-131] After the quinone is formed, multiple potential degradation reactions may occur for each individual flavonoid. The majority of these mechanisms are not in the scope of this dissertation and will not be discussed However, one particular oxidation mechanism of interest is the formation of flavan-3-ol dimers through auto-oxidative processes. Flavan-3-ol auto-oxidation dimers are classified as theasinensins (THSN) or P-2 dimers [**Figure 1.8**]. Both THSN and P-2 dimers can be further classified as homo- or hetero-dimers if their precursor monomers were two identical or two different monomeric flavan-3-ols, respectively.

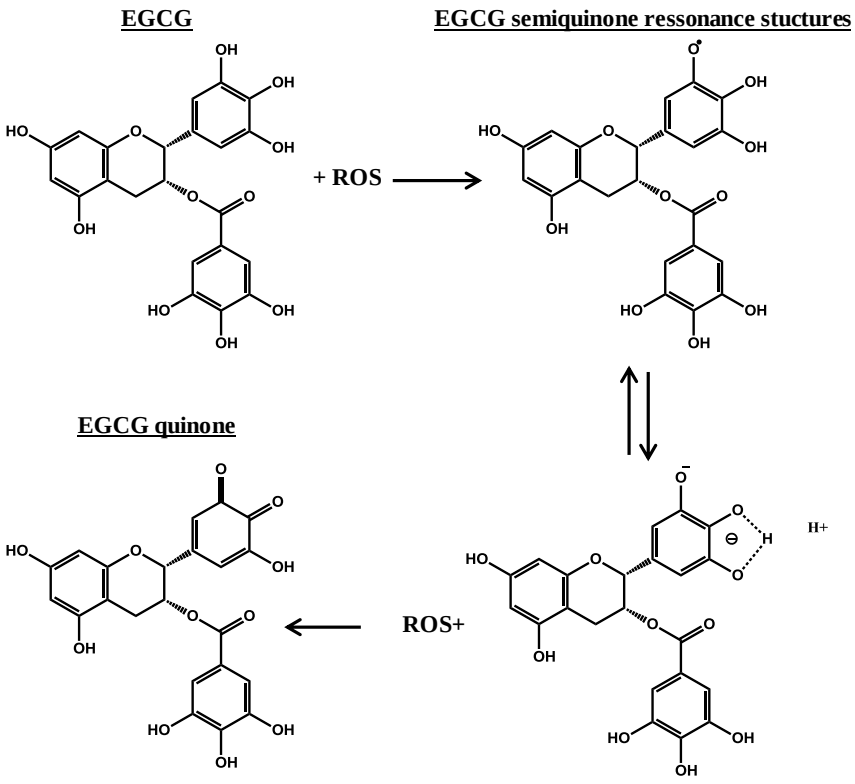
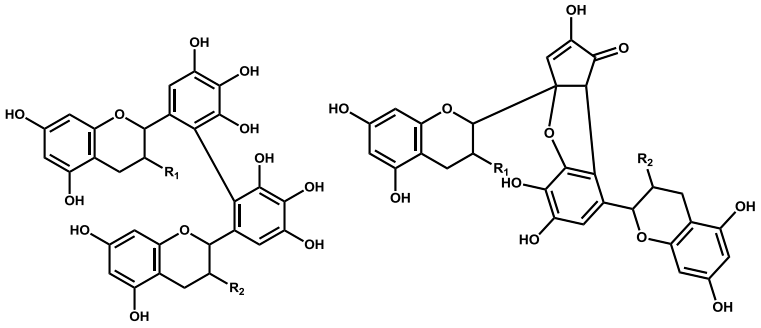


Figure 1.7 EGCG Auto-Oxidation to EGCG Quinone.
Reactive Oxygen Species (ROS) formed, multiple potential degradation reactions may occur for each individual flavonoid.



THSNs **P-2 and P-2 analogs**

Compound	Monomer Composition	m/z	R1	R2
THSN A/D	2 EGCG	913	Galloyl	Galloyl
THSN C/E	2EGC	609	OH	OH
THSN B	EGCG + EGC	761	Galloyl	OH
P-2	2 EGCG	883	Galloyl	Galloyl
P-2 analog	2 EGC	579	OH	OH
P-2 analog	EGCG + EGC	731	Galloyl/OH	OH/Galloyl

Figure 1.8 Structure of Theasinensins (THSNs) and P-2 Dimers.

Although dimers may theoretically be formed by any combination of flavan-3-ol monomers, the formation of ECG homo-dimer and EGC-ECG hetero-dimer was not observed.[132] Like most other flavonoid auto-oxidation mechanisms, the formation of flavan-3-ol quinones is a prerequisite for THSN and P-2 dimer formation.[133] Once formed, flavan-3-ol quinones may then react with flavan-3-ols or other flavan-3-ol quinones to produce a THSN dimer or its quinone analogue, respectively.[133] Additional oxidation of the THSN dimer will produce its quinone analogue as well, which can then be further oxidized to form the P-2 dimer.[133] However, the exact mechanism of P-2 dimer formation is still uncertain. For instance, Yoshino et al. demonstrated that the incubation of isolated THSN A did not form P-2 dimers.[134] This suggests that the formation of the P-2 dimer involves additional mechanistic requirements and not just the oxidation of THSN A. THSN and P-2 dimers are formed during tea fermentation or oxidation and are thus present in relatively high concentration in black teas.[29] Furthermore, the formation of dimers is not limited to food processes, but may also occur during digestion and experimental conditions.[132-135] Thus, a significant portion of monomeric flavan-3-ols may be converted to THSNs or P-2 dimers after consumption. Recently, research has focused on the absorption and the potential impact of THSNs on health. THSNs have been shown to be accumulated and transported through Caco-2 cells and absorbed by rats after oral consumption.[29, 136] Furthermore, THSNs have been shown to induce apoptosis and inhibit the expression of cyclooxygenase-2 in cell cultures.[137-138] However, the chemistry of THSNs in food systems has not been as widely researched as their monomeric counterparts. Thus, their formation and chemistry in model beverages merits further research.

1.7.2 pH Stability

It is generally agreed upon that flavonoids are more stable in acidic solutions rather than neutral or basic solutions. At elevated pHs, ortho-hydroxy moieties readily deprotonate and facilitate the formation a quinone oxidation product.[139] Flavonoids and phenolic acids without ortho-hydroxy substitutions are less susceptible to pH changes and are unable to form a quinone oxidation product.[139] Thus, the flavonol kaempferol and flavanones naringenin and hesperetin are not as susceptible to oxidization induced by elevated pHs [**Figure 1.4**]. However, a study found that naringenin and hesperetin decompose to phloroglucinol in basic conditions once exposed to extreme temperatures (300^{o}C), which is not entirely applicable to food system.[140] The majority of studies that examined the behavior of flavonoids under various pH conditions focused primarily on flavonoids composed of ortho-hydroxy B-rings, specifically anthocyanins and monomeric flavan-3-ol s.

Anthocyanins are particularly sensitive to alterations in pH. Cyanidin, delphinidin, and petunidin contain an ortho-hydroxy substitution on the B-ring, which is particularly vulnerable to oxidation. Furthermore, additional molecular modifications induced by varying pH may occur on both A and C rings. As previously discussed, anthocyanidins may be present as quinoidal bases, flavylium cations, chalcones, and pseudo-bases/hemiketals [**Figure 1.3**].[7] Because of these unique structural features, studies have focused on the behavior of anthocyanins under different conditions. Anthocyanin rich extracts including red sweet potato, purple corn, purple carrot, and red grape were found to be stable in pH 1 to pH 4 solutions.[47] Furthermore, the thermal

degradation activation energies of black carrot anthocyanins were reported as 72.4, 56.8, and 47.4 kJ/mol at pH 3.0, 5.0, and 7.0, respectively.[141] Thus, the thermal sensitivity of anthocyanins increased with increasing pH. Finally, isolated cyanidin, peonidin, delphindin, petunidin, and malvidin 3-O-glucosides were found to be relatively stable at pH 1-3 and increasingly less stable at higher pHs.[142]

The impact of pH on flavan-3-ol stability has been the focus of many studies. Flavan-3-ol s are particularly sensitive to elevated pHs due to the presence of either two or three hydroxy substituents on the B-ring [**Figure 1.2**]. This structural configuration is responsible, in part, for the relative differences in monomeric flavan-3-ol stability. In neutral solutions, EGCG and EGC are equally the least stable out of the monomeric flavan-3-ols, while ECG and EC are more stable.[143-144] Additionally, total green tea flavan-3-ols remained stable for 18 hours at pH 5, but not at pHs greater than 6.5.[143-144] Furthermore, greater than 80% of green tea flavan-3-ols remained after 20 minutes at 120^{o}C in pH 3 and 4 solutions. Approximately 50% and 20% of green tea flavan-3-ols remained after the same conditions at pH 5 and 6, respectively.[30] Other studies have focused on the interaction of specific flavan-3-ols at various pH solutions. For instance, Komatsu et al. illustrated that in acidic solutions, EC epimerization to C is the primary mechanism of degradation, not oxidation.[145] Furthermore, Ishino et al. demonstrated that epimerization of epicatechin occurs roughly fifty times more rapidly at pH 8 than pH 5 and that gallated epicatechins are more prone to epimerization.[146] This suggests that the epimerization and oxidation reactions are both induced in basic conditions, which leads is responsible for the observed pH sensitivity of flavan-3-ols.[147]

1.7.3 Temperature

At elevated temperatures, flavonoid degradation mechanisms accelerate and therefore contribute to rapid degradation. It is generally accepted that flavonoid closely follow first order thermal degradation and Arrhenius behavior. Thus, the majority of studies that investigate the impact of thermal stress on flavonoids characterize their behavior by calculating the activation energies of degradation. As the activation energy of a chemical reaction decreases, less energy is required to form a mechanistic transition state, thus enabling the reaction to proceed. This dissertation primarily focuses on the thermal degradation of anthocyanins and monomeric flavan-3-ols, thus those classes will only be discussed in detail.

The thermal degradation of anthocyanins in many different juices, extracts, and model beverages has been extensively studied. In general, first order degradation rate constants for both total monomeric and individual anthocyanins were directly proportional to increased temperature. [141, 148-155] As previously discussed, activation energies for anthocyanin thermal degradation are generally calculated by the Arrhenius equation and used to characterize their sensitivity to elevated temperature. However, it should be noted that Eyring and Ball models were also found to be accurate methods to calculate activation energies.[148] Furthermore, factors including the composition of anthocyanins, pH, and total dissolved solids alter activation energies. For instance, activation energies for purple-flesh potato, red-flesh potato, grape, and purple carrot extracts at pH 3.0 were 72.5, 66.7, 75.0, and 81.3, kJ/mol, respectively.[149] The activation energies of black carrot anthocyanins were previously reported as 72.4, 56.8, and 47.4 kJ/mol at pH 3.0, 5.0, and 7.0, respectively.[141] Additionally, activation energies of blood

orange anthocyanin degradation were previously reported as 73.6, 84.5, and 89.5 kJ/mol at 11.1, 45.0, to 69.0 ^{o}Brix, respectively.[150] Several other studies specifically investigated the thermal degradation of individual anthocyanins in solution in order to illustrate the differences caused by anthocyanidin structure and various glycosides. The thermal degradation activation energies of cyanidin, peonidin, and malvidin 3-O-glucosides were calculated to be 67.38, 57.75, and 95.39 kJ/mol at pH ~3.0.[154-155] Furthermore, cyanidin 3,5-diglucoside and 3-O-rutinoside had activation energies of 66.03 and 71.53 kJ/mol under these same conditions, respectively.[154]

The thermal degradation of green tea flavan-3-ols has been the focus of many studies and is well characterized. Tea flavan-3-ols are sensitive to heat and tend to follow either first order or pseudo-first order thermal degradation. Furthermore, the Arrhenius model is generally used to describe the accelerated degradation rate at elevated temperatures. Komatsu et al. demonstrated that the Arrhenius plots of individual flavan-3-ols is not linear and may be estimated by creating two separate linear regions.[145] Activation energies were estimated for ECG, EC, EGC, and EGCG to be 13.4, 21.8, 15.1, and 19.7 kJ/mol or 150, 159, 172, 158 kJ/mol if temperatures below or above 82 ^{o}C, respectively. [145] However, there is some disagreement as others have calculated 78.2 kJ/mol as the activation energy for EGCG.[128] As previously discussed, flavan-3-ols tend to degrade through a combination of epimerization and dimerization mechanisms. Studies have suggested elevated temperatures increase the rate of epimerization for EC, EGC, ECG, and EGCG, under various experimental conditions.[30, 156] Wang et al. conducted two elaborate experiments that detailed the kinetic modeling of EGCG and ECG degradation and epimerization.[157-158] The activation energy of EGCG and GCG

thermal degradation was calculated to be 43.09 kJ/mol.[157] Furthermore, the activation energies for EGCG to GCG and GCG to EGCG epimerization were calculated to be 105.07 and 84.33 kJ/mol, respectively.[157] Because the overall degradation of EGCG and GCG requires less energy than either epimerization reaction, this suggests that another degradation mechanisms significantly contributes to the thermal sensitivity of EGCG. It is likely that the formation of THSN A may contribute, in part, to this observation.

1.7.4 Light

Flavonoids, specifically anthocyanins, have been associated with accelerated degradation when exposed to light. For example, color parameters of wine were altered when exposed to light for 6 days, suggesting significant changes in anthocyanin concentration.[159] Furthermore, anthocyanin photo degradation may be modeled by first order kinetics.[160] In contrast to thermal degradation, the change in photo degradation reaction rate cannot be modeled by the Arrhenius equation. However it should be noted, the activation energies of cyanidin and peonidins arabinosides and galactosides were reduced by roughly 72% when exposed to light, relative to a dark condition control under identical thermal treatments. [160] Thus, the exposure to light dramatically increases the degradation rate of anthocyanins. The exact mechanism of anthocyanin photo degradation has not been identified. Furtado et al. and Dyrby et al. suggested that at least two mechanisms of photo degradation.[152, 161] The first involves the formation of a carbinol pseudobase which then forms a chalcone, while the second involves the direct formation of a chalcone. Furthermore, acylated anthocyanins were found to be more resilient to photo degradation than their non-acylated counterparts. [47, 162-164] Evidence

suggests that intramolecular copigmentation by either one or two acylated moieties significantly contribute to this observation.[48] Interestingly, several studies illustrated that acylated anthocyanin rich extracts exposed to light form new anthocyanin related pigments.[162-163] Therefore, it is likely that other photo degradation mechanism remain unidentified.

1.8 Interactions with Protein

Polyphenol-protein interactions have been the focus of many studies and investigated in relation to stability of beverages, impact through consumption, and absorption, and implications on circulation. For instance, these interactions have been identified as the primary driving factor for haze formation in beverages.[165] Evidence suggests that polyphenol to protein ratio, relative proline composition, and polyphenol structures were all partially responsible for inducing these interactions.[165-166] Furthermore, the interaction between monomeric flavan-3-ols with food proteins has been of particular interest. Thermally induced co-assemblies of EGCG and β-lactoglobulin showed a significant protective effect.[167] However, EGCG showed maximum binding affinity and binding capacity other predominant food proteins including β-casein and gelatin A, B, and C.[168] During consumption of polyphenol rich beverages, proline rich salivary proteins may bind to flavan-3-ols, resulting in the precipitation of their complex which may be responsible for an astringent sensation.[169-170] Furthermore, these interactions may play a significant role through digestion and absorption.[171-172] Flavonoids are circulated throughout the body by binding to human serum albumin.[173] Studies have indicated that flavan-3-ols specifically interact with

proline rich segments of proteins.[174-175] These interactions are primarily driven by a combination of hydrogen bonding and hydrophobic forces between proline rich segments and phenolic rings on flavan-3-ols.[176-177]

1.9 Research Aims

The chemistry of flavonoids and their interactions with environmental factors and other compounds in solution has been the focus of many studies. However in order to aid in the development of foods and beverages suitable for disease preventative dietary strategies, further research must be conducted to fully comprehend this complex chemistry. For instance, although thermal sensitivity of anthocyanins has been well documented, limited information is available concerning photo degradation and application in commercially relevant model beverage systems where extended shelf life is a primary concern. Furthermore, there is currently no hypothesized mechanism for the degradation of anthocyanins and/or formation of new pigments during the photo degradation of structurally diverse acylated anthocyanins, typically believed to be more process stable. Additionally, the interactions of green tea flavan-3-ols with proline rich proteins have been the focus of several studies relative to their ability to modulate astringent taste and bioavailability of flavonoids. However, these interactions have the potential to modulate process and shelf stability of flavonoids. To date the impact of these interactions on stability of flavonoids in model food/beverage systems remains unknown. Modulation of flavonoid oxidation and other degradative processes may alter the potential human health benefits. In an effort to develop this critical knowledge, our

overall objective is to investigate the interaction of flavonoids in model beverages and human biological fluids through varying environmental conditions.

AIM1: Investigate the impact of thermal and photo stresses on grape and purple sweet potato anthocyanins in model beverages. Our *working hypothesis* is that acylated anthocyanins in purple sweet potato will be more resilient to both thermal and photo stress conditions than non-acylated anthocyanins in grape extract. We base our hypothesis on previous studies that indicated the impact of inter- and intramolecular copigmentation interactions. Furthermore, we anticipate that interactions with ingredients in model beverages including ascorbic acid, sodium benzoate, potassium sorbate, minerals, and pH will cause significant differences in anthocyanin stability. Finally, mass spectrometry data will allow us to follow the degradation of individual anothcyanins, which may suggest some mechanistic details of degradation through various stresses.

AIM2: Explore the presence of select flavonoids and flavonoid metabolites in human milk samples donated from four cohorts of free living women through multiple lactation periods. Our *working hypothesis* is that flavonoids and their metabolites are deposited in human milk, but have no correlation to lactation stage. Furthermore, we anticipate inter- and intra-individual variability to be fairly high because the lack of dietary controls. However, we expect that some differences will be evident between cohorts as dietary habits may differ among them. Furthermore, a scan of potential

metabolites may reveal the nature of flavonoid metabolites that are deposited, stabilized in human milk and made available to infants during feeding. We base our hypotheses studies that reported the transient nature of flavonoids in other biological fluids and tissues and their interaction with milk proteins. By fully investigating these research aims, we will facilitate a deeper understanding of flavonoid chemistry in both model beverages and biological fluids that serve as primary nutrition sources for infants. Specifically, while mapping flavonoid stability, these studies are designed to investigate and suggest potential mechanisms of degradation and stabilization. Furthermore the focus on human milk draws specific attention to protein containing beverages as potential flavonoid carriers leading to **Aim 3**.

AIM3: Investigate the impact of milk protein concentrations on the stability of green tea flavan-3-ols and the formation of THSN and P-2 dimers. Our *working hypothesis* is that greater polyphenol to protein ratios will increase flavan-3-ol degradation rates. In addition, we hypothesize that galloylated flavan-3-ols (EGCG and ECG) degradation rates will significantly decrease with increasing concentrations of protein. In contrast, we expect non-galloylated flavan-3-ols (EC and EGC) degradation rates not to be significantly altered by protein concentrations. These effects primarily driven by the increased protein binding associated with gallated versus non gallated flavan-3-ols. Furthermore, we anticipate that THSN and P-2 dimer formation will accelerate at higher polyphenol to protein ratios. We base our hypotheses on previous studies that indicated that relative binding of polyphenols to milk proteins and the formation of auto-oxidation dimers. Finally, by using a wide range of flavan-3-ol

concentrations, we may be able approximate the interactions that may occur between polyphenols and proteins in biological fluids.

CHAPTER 2. PHOTO AND THERMAL DEGRADATION OF ANTHOCYANINS FROM GRAPE AND PURPLE SWEET POTATOIN MODEL BEVERAGE SYSTEMS

2.1 Introduction

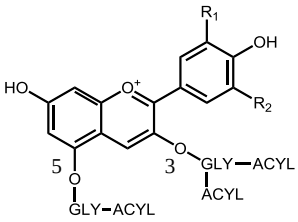
With increasing public awareness of the potential health issues associated with synthetic food color additives, the food industry became particularly interested in replacing these additives with natural alternatives, including fruit and vegetable extracts.[178-179] Anthocyanins are a group of natural pigments responsible for the bright blue and red colors found in many flowers, fruits, and vegetables. They can be classified based on the presence of hydrogen, hydroxyl, or methoxy substituents on the aglycone **[Figure 2.1]**. Naturally, anthocyanidins are glycosylated with sugar moieties, including but not limited to glucoside, galactoside, sophoroside, rutinoside, and sambubioside, attached to the aglycone at the 3 position for monoglycosides and the 3,5 positions for diglycosides.[8] Anthocyanins may also be acylated with the presence of moieties including phenolic acids such as ferulic, p-hydroxybenzoic and caffeic acids on their glycosides.[9]

In addition to serving as potential natural alternatives to the synthetic color additives, anthocyanins have been reported to promote disease preventative properties. Reactive oxygen species are a concern to human health because of their ability to promote oxidative stress, age-related dysfunction, and damage to overall cardiovascular

Adapted from Song, B. J.; Sapper, T. N.; Burtch, C. E.; Brimmer, K.; Goldschmidt, M.; Ferruzzi, M. G., Photo- and Thermodegradation of Anthocyanins from Grape and Purple Sweet Potato in Model Beverage Systems. *Journal of Agricultural and Food Chemistry* **2013**, 61, 1364-1372, with permission.

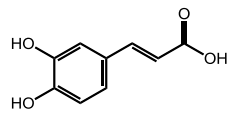
health.[180] Additionally, research has shown that dietary phytochemicals, including anthocyanins found in red wines, are capable of scavenging free radicals *in vitro*.[46, 181] For instance, anthocyanins from fruits and vegetables including strawberries and grapes reduce the risk of neurodegenerative diseases by mitigating oxidative damage in cell culture models.[182-183]

While promising as health promoting natural colors, anthocyanins are susceptible to a diverse array of chemical reactions and are notoriously sensitive to heat, light, and oxygen. Thus, if anthocyanins are broadly applied in the replacement of synthetic food color additives, their stability to conditions of thermal processing, handling, and storage in the context of specific formulations must be investigated. Many previous studies focused on the degradation kinetics of anthocyanins in relation to their color, but not the anthocyanin compositions or concentrations.[47, 184] Other studies examined the degradation kinetics of total monomeric anthocyanins in juices and concentrates.[151, 185-186] However, few studies examined the total monomeric anthocyanin content and individual

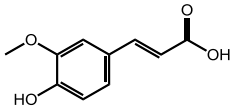


	R_1	R_2
Cyanidin	OH	H
Dephinidin	OH	OH
Malvidin	OCH_3	OCH_3
Peonidin	OCH_3	H
Petunidin	OH	OCH_3

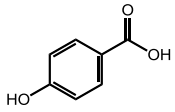
Acylated Moieties



Caffeic acid



Ferulic acid



p-hydroxybenzoic acid

Figure 2.1 Structures of Anthocyanin Backbones and Acylated Moieties Found in PSP. "GLY" (position 3 and 5) and "ACYL" represent glycosides and acylated moieties, respectively.

anthocyanin degradation kinetics in ready to drink (RTD) model beverages. Thermal stress has been the focus of many of these studies, but there is a lack of publications concerning the degradation kinetics of anthocyanins through photo stress and its implication on beverage shelf life. Furthermore, a significant portion of RTD beverages are acidified and either formulated with preservatives or thermally processed by high temperature short time (HTST) treatment in both hot fill hold or aseptically filled. Fortification of RTD beverages with vitamins and minerals has also increased and may promote the degradation of anthocyanins. The objective of this study was therefore to determine the stability of anthocyanins in grape and PSP extracts in multiple models of RTD beverages through photo and thermal stresses.

2.2 Materials and Methods

2.2.1 Materials

Ascorbic acid (AA), citric acid, sodium citrate, sodium benzoate, and potassium sorbate were obtained from Sigma Aldrich (St. Louis MO). Formic acid, LC/MS grade water and acetonitrile, potassium chloride, hydrochloric acid, and sodium acetate were purchased from VWR (Radnor, PA). Sensient Colors Ltd. (St. Louis MO) graciously donated a model vitamin enriched water premix powder, rubired grape, and purple sweet potato extracts. The vitamin enriched water premix powder contained 25 mg, 0.83 mg, 0.17 mg, 0.25 μg, 0.83 mg, 0.62 mg, and 5 μg of vitamin C, B3, B6, B12, B5, zinc(II) and chromium(III) in a 100 mL solution, respectively. Authentic standards of delphinidin, malvidin, cyanidin, petunidin, and peonidin glucosides were purchased from Chromadex Inc. (Irvine, CA).

2.2.2 Model Beverages

Model RTD beverages were created to mimic typical compositions commonly used in the beverage industry. This included a vitamin enriched water model beverage (pH=3.60 ± 0.02), a hot-fill model beverage (pH 3.60 ± 0.02) with varying AA concentrations, and a preserved model beverage (pH=3.00 ± 0.02) containing sodium benzoate and potassium sorbate. The vitamin enriched water (VEW) model was formulated with 0.025% AA and 0.044% of a model vitamin water powdered premix in deionized water. Hot fill model beverages were formulated with 0.00, 0.01, or 0.05% AA and also contained 1.60% citric acid and 1.00% sodium citrate in deionized water. A preserved model beverage was formulated with 0.05% sodium benzoate, 0.01% potassium sorbate, 2% citric acid, and 0.60% sodium citrate in deionized water. Both rubired grape and PSP extracts were dosed into the VEW model beverage to illustrate the differences in extracts rich in acylated and non-acylated anthocyanins, respectively. Rubired grape was added into all other model beverages to evaluate the stability of non-acylated anthocyanins across all model beverages. 0.11% and 0.04% of rubired grape and PSP extract were used throughout the experiments, respectively. Extract concentrations were selected based on matching color profiles provided by Sensient Ltd.

2.2.3 Photo and Thermal Stresses

After each model beverage was created, an aliquot of each solution was transferred into clear borosilicate glass tubes, flushed with nitrogen, and hermetically sealed. Thermal stress was induced by using a Precision Scientific Thelco (Chicago, IL) water bath set at 40, 60, and 80 ^{o}C for all model beverages. Photo stress was induced by

using an Atlas Material Testing Technology LLC. Suntest (Chicago, IL) lightbox equipped with a xenon lamp at 250, 500, and 750 W/m^2 (equivalently 47.1, 95.1, and 143.1 klx, respectively). The energy emitted from indoor lighting, full daylight, and direct sunlight are broadly estimated at 1, 10, and 100 klx, with variations caused by other environmental factors. Thus, the 250 W/m^2 treatment roughly simulates indoor lighting at 47 times an accelerated rate. Furthermore, the 500 W/m^2 approximates the energy a beverage may be exposed through direct sunlight. Vitamin enriched water samples were retrieved at 0, 15, 30, 60, 120, 240, and 360 min of stress while all other model beverages were retrieved at 0, 60, 120, 240, 360, 480, 600, 720, and 1440 min.

2.2.4 Anthocyanin Analysis

All beverage samples were analyzed for anthocyanin content by pH differential total monomeric anthocyanin content and LC-MS. The pH differential technique was used as described by Giusti et al. [7], with minor volume modifications to fit a 96 well plate reader. Very briefly, 60 µL of each solution was transferred into two separate wells containing either 0.24 mL of 0.025 M potassium chloride buffer (pH=1.00) or 0.4 M sodium acetate buffer (pH=4.50). After incubating at room temperature for 15 min, their absorptions were recorded at 510 and 700 nm using a Molecular Devices Spectra Max 100 (Sunnyvale, CA). Total monomeric anthocyanins were calculated based on cyanidin 3-glucoside equivalents.

Prior to all LC-MS analyses, samples were prepared by solid phase extraction. Waters Oasis® HLB 1cc Cartridges (Milford, MA) were prepared by sequentially rinsing with methanol and water. Model beverage samples were loaded onto each cartridge and

rinsed with 2% formic acid in water. Anthocyanins were eluted with 2% formic acid in methanol. Samples were dried in a Labconco RapidVap (Kansis City, MO) and solubilized in 200 µL of 95:5 mobile phases A and B.

Samples were injected in a Waters 2695 Separations Module (Milford, MA) equipped with a Waters Xterra reverse phase C18 3.5 µm 2.1x100 mm column (Milford, MA) in a heated chamber set at 35 ^{o}C. A linear biphasic gradient with a flow rate of 0.30 mL/min with mobile phase A, 2% formic acid in MS grade water, and mobile phase B, 0.1% formic acid in acetonitrile. At 0, 10, 30, and 31 min, the solvent composition was 95, 90, 75, and 95% mobile phase A. Directly tandem to the column, the flow was split so that half of it went to a Waters 2996 photodiode array detector (Milford, MA) and a Waters Micromass ZQ mass spectrometer (Milford, MA). Positive mode electrospray ionization was used with capillary, cone, and extractor voltages set to 2500, 50, and 3 volts, respectively. Source and desolvation temperatures were set to 150 and 300 ^{o}C, respectively. Nitrogen gas cone and desolvation flow rates were set to 25 and 250 L/hr, respectively. Single ion responses were set to 303.8, 331.7, 287.5, 317.7, and 301.7 m/z to detect delphinidin, malvidin, cyanidin, petunidin, and peonidin, respectively.

Acylated anthocyanins were identified and analyzed by a LC-time of flight (TOF) MS. Samples were injected into a Hewlett Packard Series 1100 LC system (Palo Alto, CA) connected to a Waters LCT Premier (Milford, MA). The LC-MS method, column, and MS conditions were identical to what was previously described for the Waters 2695 Separations Module (Milford, MA). However, the cone voltages varied from 30, 50, and 70 V in order to fragment acylated anthocyanins and aid in identification.

2.2.5 Kinetic Models

Previous studies have shown that anthocyanins typically follow first order kinetics and Arrhenius behavior through thermal stress.[151, 153, 185-186] Reaction rate constants for both total monomeric and individual anthocyanins were calculated by creating a plot of the natural log of anthocyanin concentration at a given time (C) divided by the initial anthocyanin concentration (C_o) as a function of treatment time **[Figures 2.3, 2.4, and Appendix C]**. Furthermore, activation energies (E_a) for thermal treatments were calculated by solving for $-E_a/R$ via a linear plot of the natural log of each degradation rate versus inverse temperature.

2.2.6 Experimental Design and Statistical Analysis

The study was split into four different experiments, two focused on the degradation rates of total monomeric anthocyanins and the others focused on the degradation rates of individual anthocyanins. Within each set experiments, they were further segregated by their exposure to either thermal or photo stress. Correlations between photo and thermal stress groups would be particularly difficult to interpret given that their degradation mechanisms are different. The total monomeric anthocyanin degradation rate experiments followed 3 x 5 factorial design, where three intensity levels of thermal/photo treatment were select for all five model beverages. Similarly, individual anthocyanins degradation rate experiments followed a 3 x 5 x 5, where five individual anthocyanidin glucosides are additional variables. Degradation rate constants are expressed as mean ± standard error of the mean. Group differences within each

experiment were determined by analysis of variance and Tukey's test (α=0.05) using Statistical Analysis Systems 9.2 (SAS Institute). Each model beverage was run as triplicates for each of the photo and thermal conditions.

2.3 Results and Discussion

2.3.1 Anthocyanin Profiles

Qualitative and quantitative anthocyanin profiles of grape and PSP extracts were noticeably different. Total monomeric anthocyanin concentrations were 26.44 and 15.64 cyanidin 3-O-glucosides equivalents g/L in grape and PSP extracts, respectively. Chromatograms for both grape and PSP corresponding to each aglycone m/z were analyzed **[Figure 2.2]**. Grape extract primarily consisted of non-acylated 3-O-glucosides of delphinidin, malvidin, cyanidin, petunidin, and peonidin. In contrast, PSP extract primarily contained mono and diacylated 3-sophoroside-5-glucosides of peonidin and cyanidins **[Table 2.1]**.

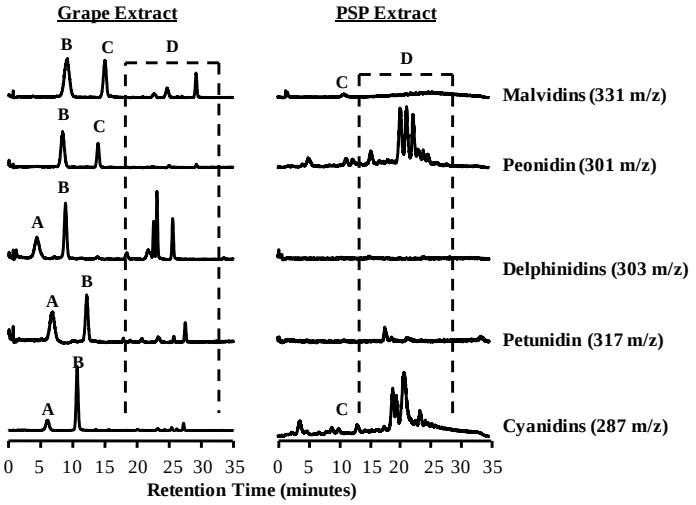


Figure 2.2 Normalized Extracted Ion Chromatograms for Grape and PSP.
Peaks labeled A-D correspond to 3,5-diglucosides, 3-O-glucosides, unidentified anthocyanins, and acylated anthocyanins, respectively, of each of the indicated aglycone chromatograms.

2.3.2 Impact of Thermal Stress on Grape Anthocyanin Degradation Rates

The total monomeric anthocyanin degradation rates for model beverages containing grape extract at 80 ^{o}C were significantly greater than both 40 ^{o}C and 60 ^{o}C treatments **[Table 2.2]**. For example, the degradation rates were $3.38x10^{-4}$, $4.84x10^{-4}$, and $1.00x10^{-3}$ min^{-1} at 40, 60, and 80 ^{o}C when incubated in the 500 ppm AA hot fill model beverage, respectively. As previously mentioned, anthocyanin degradation typically follows first order kinetics and Arrhenius behavior, which is likely to be responsible for this observation. Kechinski et al. previously reported that total monomeric anthocyanin content in blueberry juice had very similar degradation rates of

Table 2.1 Major Anthocyanin Species Present in Grape and PSP Extracts

Grape Extract			
Compound	Relative Conc. (%)	Parent Ion (m/z)	Fragment (m/z)
Malvidin 3-O-glucoside	25.8	493	331
Peonidin 3-O-glucoside	9.8	463	301
Delphinidin 3,5-diglucoside	3.1	627	303
Delphinidin 3-O-glucoside	2.2	465	303
Petunidin 3,5-diglucoside	3.5	641	317
Petunidin 3-O-glucoside	1.7	479	317
Cyanidin 3,5-diglucoside	8.9	611	287
Cyanidin 3-O-glucoside	17.1	449	287
PSP Extract			
Tentative Identification	Relative Conc.(%)	Parent Ion (m/z)	Fragment (m/z)
Peonidin-3-dicaffeoylsophoroside-5-glucoside	12.1	1111	949, 463, 301
Peonidin-3-caffeoyl-p-hydroxybenzoyl -sophoroside-5-glucoside	18.5	1069	907, 787, 625, 463, 301
Peonidin-3-caffeoyl-feruloylsophoroside-5-glucoside	14.5	1125	963, 463, 301
Peonidin-3-caffeoylsophoroside-5-glucoside	6.3	949	787, 463, 301
Peonidin-3-p-hydroxybenzoylsophoroside-5-glucoside	3.7	907	301
Peonidin-3-feruloylsophoroside-5-glucoside	2.5	963	301
Total acylated cyanidin-3-sophoroside-5-glucosides	34.5	-	611,449,287

Relative concentrations are represented mole of anthocyanin / total anthocyanin moles in each extract. Identities of glucosides in grape extracts were confirmed by analytical standards and mass spectroscopy. Tentative identifications were based on previous studies on PSP acylated anthocyanins, parent ions, and fragment ions **[Appendix A]**.[9, 187]

$6.40x10^{-5}$, $4.57x10^{-4}$, and $2.25x10^{-3}$ min^{-1} at 40, 60, and 80 °C, respectively.[151] Furthermore, the increase in thermal treatment resulted in similar significant differences for all anthocyanidin 3-O-glucosides in the grape extract **[Table 2.3]**. Delphinidin, malvidin, petunidin, peonidin, and cyanidin 3-O-glucosides are predominant anthocyanin species in grape extract. Thus, the degradation rates for both total monomeric anthocyanins and anthocyanidin 3-O-glucosides are likely to follow similar significant differences across thermal treatments.

Interestingly, negative values for degradation rates were calculated for individual anthocyanins in the 0 ppm AA hot fill and preserved model beverages **[Table 2.3]**. Although this may imply that these anthocyanidin 3-O-glucosides are increasing in concentration, this is highly unlikely given the conditions of these experiments. Negative degradation rates were only observed in the most stable model beverages coupled with the mildest thermal treatment, suggesting only minimal changes to anthocyanin content.

Table 2.2 Total Monomeric Anthocyanin Degradation Rates and Activation Energies Under Thermal Stress

Model Beverage	40 °C (min^{-1})	60 °C (min^{-1})	80 °C (min^{-1})	E_a (kJ/mol)
0 ppm AA Hot Fill	1.36E-5 ± 4.8E-5^{1A}	1.71E-4 ± 3.3E-5^{2A}	9.43E-4 ± 1.2E-5^{3A}	60.48 ± 3.3^{AB}
100 ppm AA Hot Fill	3.34E-4 ± 3.9E-5^{1B}	3.32E-4 ± 6.9E-5^{1AB}	9.69E-4 ± 2.3E-5^{2A}	24.20 ± 2.8^{C}
500 ppm AA Hot Fill	3.38E-4 ± 2.0E-5^{1B}	4.84E-4 ± 6.5E-5^{1B}	1.00E-3 ± 2.0E-5^{2A}	24.78 ± 1.8^{C}
VEW (Grape)	3.02E-4 ± 1.1E-5^{1B}	5.02E-4 ± 2.9E-5^{1B}	1.88E-3 ± 6.1E-5^{2B}	41.60 ± 0.2^{BC}
Preserved Model	2.41E-5 ± 2.4E-5^{1A}	1.59E-4 ± 2.2E-5^{1A}	1.10E-3 ± 8.6E-5^{2A}	80.07 ± 8.6^{A}

Ascorbic acid and vitamin enriched water are abbreviated AA and VEW, respectively. VEW (PSP) total monomeric anthocyanin degradation rates are not shown because of their deviation from first order kinetics. All model beverages contained grape extract. VEW model beverage (pH=3.60 ± 0.02) contained 0.025% AA and 0.044% vitamin water powdered premix. All hot filled model beverages (pH 3.60 ± 0.02)contained 0.60% citric acid and 1.00% sodium citrate, with various concentrations of AA indicated in the table. Preserved model beverage (pH=3.00 ± 0.02) contained 0.05% sodium benzoate, 0.01% potassium sorbate, 2% citric acid, and 0.60% sodium citrate. Different superscript numbers indicate significant differences ($P<0.05$) between temperatures within each model beverage. Different superscript letters indicate significant differences ($P<0.05$) between model beverages within each temperature or activation energy.

Furthermore, negative values were only obtained when the absolute value of the mean was less than the standard error of the mean **[Table 2.3]**. This is likely due to the inability of the LC-MS analysis of individual anthocyanins to distinguish the extremely low degradation from analytical noise. However, because the pH differential method measures total monomeric anthocyanins, it was more capable of assessing broad changes in the degradation of total anthocyanins throughout the thermal treatment.

Table 2.3 Grape Anthocyanin Degradation Rates and Activation Energies Under Thermal Stress.

0 ppm AA Hot Fill				
	40 ^{o}C (min^{-1})	60 ^{o}C (min^{-1})	80 ^{o}C (min^{-1})	E_a (kJ/mol)
Delphinidin 3-O-glucoside	2.18E-4 ± 2.3E-4$^{1A\alpha}$	8.82E-4 ± 8.3E-5$^{1A\alpha}$	1.89E-3 ± 1.0E-4$^{2A\alpha}$	30.29 ± 1.1$^{A\alpha}$
Malvidin 3-O-glucoside	-4.50E-5 ± 1.0E-4$^{1A\alpha}$	1.87E-4 ± 6.6E-5$^{1A\alpha}$	1.51E-3 ± 6.4E-5$^{2A\alpha}$	67.52 ± 0.6$^{B\alpha}$
Petunidin 3-O-glucoside	-1.65E-5 ± 7.4E-5$^{1A\alpha}$	2.21E-4 ± 5.2E-5$^{1A\alpha}$	1.42E-3 ± 3.9E-4$^{2A\alpha}$	65.77 ± 6.7$^{B\alpha}$
Peonidin 3-O-glucoside	-3.67E-5 ± 1.1E-4$^{1A\alpha}$	1.93E-4 ± 4.7E-5$^{1A\alpha}$	1.31E-3 ± 3.7E-5$^{2A\alpha}$	59.97 ± 0.4$^{B\alpha}$
Cyanidin 3-O-glucoside	1.65E-5 ± 3.6E-5$^{1A\alpha}$	3.36E-4 ± 9.1E-6$^{1A\alpha}$	1.64E-3 ± 5.3E-5$^{2A\alpha}$	75.71 ± 0.7$^{B\alpha}$
100 ppm AA Hot Fill				
	40 ^{o}C (min^{-1})	60 ^{o}C (min^{-1})	80 ^{o}C (min^{-1})	E_a (kJ/mol)
Delphinidin 3-O-glucoside	3.04E-4 ± 1.6E-4$^{1A\alpha}$	6.49E-4 ± 2.7E-5$^{1A\alpha}$	1.54E-3 ± 1.9E-4$^{2A\alpha}$	43.12 ± 11.7$^{A\alpha}$
Malvidin 3-O-glucoside	3.51E-4 ± 6.9E-6$^{1A\alpha\beta}$	2.74E-4 ± 2.1E-5$^{1A\alpha}$	1.31E-3 ± 1.4E-4$^{2A\alpha}$	29.19 ± 3.0$^{A\beta}$
Petunidin 3-O-glucoside	3.51E-4 ± 9.7E-5$^{1A\alpha\beta}$	2.99E-4 ± 1.1E-5$^{1A\alpha}$	1.53E-3 ± 1.7E-4$^{2A\alpha}$	34.12 ± 4.1$^{A\beta}$
Peonidin 3-O-glucoside	3.13E-4 ± 8.4E-5$^{1A\alpha\beta}$	1.68E-4 ± 9.4E-6$^{1A\alpha}$	1.11E-3 ± 2.2E-4$^{2A\alpha}$	28.58 ± 1.9$^{A\beta}$
Cyanidin 3-O-glucoside	3.61E-4 ± 2.1E-5$^{1A\alpha\beta}$	3.81E-4 ± 1.3E-6$^{1A\alpha}$	1.47E-3 ± 1.6E-4$^{2A\alpha}$	31.49 ± 3.9$^{A\beta}$
500 ppm AA Hot Fill				
	40 ^{o}C (min^{-1})	60 ^{o}C (min^{-1})	80 ^{o}C (min^{-1})	E_a (kJ/mol)
Delphinidin 3-O-glucoside	6.83E-4 ± 1.5E-5$^{1A\alpha}$	1.11E-3 ± 5.8E-5$^{1A\alpha}$	1.70E-3 ± 4.9E-5$^{2A\alpha}$	20.93 ± 1.2$^{A\alpha}$
Malvidin 3-O-glucoside	5.16E-4 ± 4.9E-5$^{1A\alpha\beta}$	7.44E-4 ± 1.1E-4$^{1A\alpha\beta}$	1.23E-3 ± 1.3E-4$^{2A\alpha}$	19.89 ± 0.4$^{A\beta}$
Petunidin 3-O-glucoside	5.96E-4 ± 2.9E-5$^{1A\alpha\beta}$	8.92E-4 ± 9.4E-5$^{1A\alpha\beta}$	1.69E-3 ± 6.0E-5$^{2A\alpha}$	23.81 ± 1.8$^{A\beta}$
Peonidin 3-O-glucoside	5.42E-4 ± 5.1E-5$^{1A\alpha\beta}$	7.94E-4 ± 9.2E-5$^{1A\alpha\beta}$	1.40E-3 ± 3.9E-5$^{2A\alpha}$	21.84 ± 2.7$^{A\beta}$
Cyanidin 3-O-glucoside	6.64E-4 ± 1.2E-5$^{1A\beta}$	9.57E-4 ± 7.9E-5$^{1A\beta}$	1.62E-3 ± 4.6E-5$^{2A\alpha}$	20.43 ± 1.0$^{A\beta}$

Table 2.3 Continued

Vitamin Enriched Water				
	40 °C (min^{-1})	60 °C (min^{-1})	80 °C (min^{-1})	E_a (kJ/mol)
Delphinidin 3-O-glucoside	5.04E-4 ± 5.7E-4$^{1A\alpha}$	1.36E-3 ± 3.8E-4$^{1A\alpha}$	4.57E-3 ± 2.9E-4$^{2A\beta}$	29.91 ± 1.2$^{A\alpha}$
Malvidin 3-O-glucoside	7.70E-4 ± 2.3E-4$^{1A\beta}$	1.06E-3 ± 8.9E-5$^{1A\beta}$	3.21E-3 ± 7.1E-5$^{2A\beta}$	34.09 ± 6.9$^{A\beta}$
Petunidin 3-O-glucoside	1.03E-3 ± 1.8E-4$^{1A\beta}$	1.28E-3 ± 1.4E-5$^{1A\beta}$	4.01E-3 ± 1.8E-4$^{2A\beta}$	31.15 ± 3.0$^{A\beta}$
Peonidin 3-O-glucoside	7.87E-4 ± 2.3E-4$^{1A\beta}$	1.14E-3 ± 5.4E-5$^{1A\beta}$	3.81E-3 ± 1.6E-4$^{2A\beta}$	37.34 ± 6.2$^{A\beta}$
Cyanidin 3-O-glucoside	1.08E-3 ± 5.7E-5$^{1A\delta}$	1.30E-3 ± 4.0E-5$^{1A\beta}$	4.55E-3 ± 5.5E-5$^{2A\beta}$	26.89 ± 0.7$^{A\beta}$
Preserved Model				
	40 °C (min^{-1})	60 °C (min^{-1})	80 °C (min^{-1})	E_a (kJ/mol)
Delphinidin 3-O-glucoside	-1.48E-5 ± 7.8E-5$^{1A\alpha}$	1.57E-4 ± 8.1E-6$^{1A\alpha}$	2.25E-3 ± 1.2E-4$^{2A\alpha}$	75.39 ± 1.3$^{A\beta}$
Malvidin 3-O-glucoside	-5.35E-5 ± 8.4E-5$^{1A\alpha}$	7.67E-5 ± 1.8E-5$^{1A\alpha}$	9.37E-4 ± 3.1E-6$^{2A\alpha}$	66.37 ± 0.1$^{A\alpha}$
Petunidin 3-O-glucoside	-1.69E-5 ± 9.8E-5$^{1A\alpha}$	1.53E-4 ± 2.4E-5$^{1A\alpha}$	1.35E-3 ± 6.6E-5$^{2A\alpha}$	58.09 ± 1.3$^{A\alpha}$
Peonidin 3-O-glucoside	-5.16E-5 ± 9.1E-5$^{1A\alpha}$	8.23E-5 ± 2.1E-5$^{1A\delta}$	9.16E-4 ± 8.2E-5$^{2A\alpha}$	60.88 ± 2.0$^{A\alpha}$
Cyanidin 3-O-glucoside	-1.76E-5 ± 6.0E-5$^{1A\alpha}$	8.03E-5 ± 4.6E-5$^{1A\alpha}$	1.03E-3 ± 7.2E-5$^{2A\delta}$	65.18 ± 0.9$^{A\alpha}$

VEW model beverage (pH=3.60 ± 0.02) contained 0.025% ascorbic acid (AA) and 0.044% vitamin water powdered premix. All hot filled model beverages (pH 3.60 ± 0.02) contained 0.60% citric acid and 1.00% sodium citrate, with various concentrations of AA indicated in the table. Preserved model beverage (pH=3.00 ± 0.02) contained 0.05% sodium benzoate, 0.01% potassium sorbate, 2% citric acid, and 0.60% sodium citrate. Different superscript numbers indicate significant differences ($P<0.05$) between temperatures within each model beverage and anthocyanin. Different superscript Roman letters indicate significant differences ($P<0.05$) between anthocyanins within each temperature and model beverage. Different superscript Greek letters indicate significant differences ($P<0.05$) between model beverages within each temperature and anthocyanin.

2.3.3 Impact of Model Beverages on Anthocyanin Degradation Rates Through Thermal Stress

Increasing concentrations of AA had varying impacts on total monomeric anthocyanin degradation rates **[Table 2.2]**. At 40 °C, degradation rates significantly increased from $1.36x10^{-5}$ to $3.34x10^{-4}$ min^{-1} with the addition of 100 ppm AA. However, the further addition of 400 ppm AA caused no significant increase in the degradation rate. At 60 °C there was only a significant increase in degradation rates between 0ppm and 500 ppm AA and no significant difference at 80 °C for any of the three hot fill model beverages. Like anthocyanins, AA is vulnerable to high temperatures and may rapidly

degrade in the model beverages [188]. Thus, the specific effect of AA on anthocyanin degradation may diminish at elevated temperature. Therefore, AA only caused significant differences in anthocyanin degradation rates at low temperatures and its effect became more negligible at higher temperatures. Previous studies have shown that the addition of AA reduces anthocyanin stability at lower temperatures. For instance, Calvi and Francis found that the addition of AA to model beverages increased the rates of degradation at 37.7, 23.8, and 1.1 ^{o}C [189]. However, unlike the present study, they did not investigate the diminishing effect of AA on anthocyanin degradation at elevated temperatures including 60 and 80 ^{o}C. Many beverages are exposed to high temperatures during processing, thus the interaction between temperature, AA concentration, and anthocyanin degradation must be considered in beverage production.

Varying AA concentrations between hot fill model beverages did not significantly increase most anthocyanidin 3-O-glucoside degradation rates at a given temperature **[Table 2.3]**. The only exception was cyanidin 3-O-glucoside, where the degradation rate significantly increased from $1.65x10^{-5}$ to $6.64x10^{-4}$ min^{-1} and $3.36x10^{-4}$ to $9.57x10^{-4}$ min^{-1} with the addition of 500 ppm AA at 40 and 60 ^{o}C, respectively. Cyanidin 3-O-glucoside is particularly sensitive to AA relative to the other anthocyanidin 3-O-glucosides present in the grape extract. Therefore, the significant increase in total monomeric anthocyanin degradation rates discussed previously may have been predominantly driven by cyanidin 3-O-glucoside. Talcott et al. examined the effect of the addition of 800 ppm AA on red muscadine grape anthocyanin 3,5-diglucosides through pasteurization (95 ^{o}C for 15 min).[190] In contrast to our findings, anthocyanidins had a significant impact on anthocyanidin 3,5-diglucoside stability. For instance, significant differences were

observed for cyanidin, pelargonin, peonidin, and malvidin 3,5-diglucosides; but not delphinidin and petunidin 3,5-diglucoside. It is likely that 800 ppm AA was concentrated enough to significantly impact most anthocyanin degradation rates, while 500 ppm was only enough to cause particularly sensitive anthocyanins to significantly reduce stability.

There was no significant difference in total monomeric degradation rates between the VEW and the 500 ppm AA model beverages at 40 ^{o}C and 60 ^{o}C. However, the VEW had a significantly greater degradation rate at 80 ^{o}C **[Table 2.2]**. Similarly, degradation rates for all anthocyanidin 3-O-glucosides were significantly greater in the VEW than the 500 ppm AA model beverage at 80 ^{o}C **[Table 2.3]**. It is likely that the increase in degradation rates is caused by the presence of metal ions and B vitamins in the VEW premix. Zinc (II) and chromium (III), which is present in the VEW model beverage, may act as an oxidizing agent and may have contributed to anthocyanin oxidation, leading to more rapid degradation. B vitamins are typically thermally stable and their interactions with anthocyanins have yet to be explored individually. Complex model beverages and juices contain many different compounds and anthocyanin degradation rates are particularly difficult to compare between studies. Thus, further research is required to determine the impact of the food ingredients on anthocyanin degradation.

2.3.4 Arrhenius Behavior and Activation Energies

The exponential increase in degradation rates with elevated temperatures is typical of Arrhenius behavior and has been reported in several other studies.[*151, 153, 185-186*] Activation energies for the degradation of total monomeric anthocyanins were calculated for each solution **[Table 2.2]**. The 0ppm AA and preserved model beverages had the

greatest activation energies at 60.48 and 80.07 kJ/mol, respectively, indicating that they are the most resilient to thermal stress. Dybry et al. investigated grape skin anthocyanin stability in a pH 3.0 buffer and calculated an activation energy of 58 kJ/mol.[152] This is in agreement with the activation energy of the 0 ppm AA hot fill model beverage (pH 3.6) used in this study. Furthermore, the addition of at least 100 ppm AA significantly reduced the activation energy to 24.20 kJ/mol. However, an additional 400 ppm did not significantly alter the activation energy. Therefore, AA concentrations of at least 100 ppm in a beverage will significantly reduce the anthocyanin degradation activation energy and thus change the response of the degradation rates between temperatures. Additional studies are required to investigate the change in activation energies with the addition of AA at lower concentrations than 100 ppm. Multiple mechanisms for degradative reactions between ascorbic acid and anthocyanins have been hypothesized. One mechanism involved the formation of reactive oxygen species, such as peroxide, from the oxidation of ascorbic acid and their subsequent reaction with anthocyanins.[191-192] Another proposed mechanism involves a condensation reaction between ascorbic acid and anthocyanins.[193] While products of individual anthocyanin-ascorbic acid condensation were not observed in the present study, it is important to highlight that the analytical conditions applied were likely not optimized for these analysis. Therefore, it remains unclear which reactions may predominate in the model beverages and conditions investigated in the present study. Additional studies into the specific mechanisms are therefore warranted.

Typically, activation energies of the anthocyanins were not significantly different in each model beverage **[Table 2.3]**. A study by Attoe and Von Elbe found no significant

difference between the activation energies of cyanidin and peonidin 3-arabinosides and 3-galatosides in a pH 2.5 buffer during dark conditions.[160] However, in the present study, delphinidin 3-O-glucoside in the 0 ppm AA hot fill model was the only exception and had a significantly lower activation energy of 30.29 kJ/mol in comparison all other anthocyanins, indicating that it is the most sensitive to temperature changes. Additionally, delphinidin 3-O-glucoside had no significant change in activation energy with the addition of ascorbic acid or vitamin premix. However, in the preserved base, delphinidin 3-O-glucoside activation energy significantly increased to 75.39 kJ/mol, highlighting the stability of the preserved base. Activation energies of malvidin, petunidin, peonidin, and cyanidin 3-O-glucosides significantly decreased with the addition of 100 ppm AA, but not with an additional 400 ppm AA. For instance, in the 0 ppm, 100 ppm, and 500 ppm AA bases, cyanidin 3-O-glucoside had activation energies of 75.7, 31.49, and 20.43 kJ/mol, respectively. These observations agree with the significant differences in total monomeric anthocyanin degradation activation energies and provide further evidence for the impact of AA on anthocyanin stability through thermal stress.

2.3.5 Grape and Purple Sweet Potato Anthocyanins Under Thermal Stress

Total monomeric anthocyanin degradation rates of PSP and grape extracts under thermal stress could not be compared. It has been well documented that the total monomeric anthocyanin degradation of grape extract follows first order kinetics. However, extracts containing relatively high concentrations of acylated anthocyanins, like PSP, do not fit a first order degradation model through short term thermal treatments **[Figure 2.3]**.[152, 194] Correlation coefficients for PSP extract in the VEW model were 0.24,

0.06, and 0.14 at 40, 60, and 80 ^{o}C, respectively. Our results agree with previous short term thermal stability studies on acylated anthocyanin rich extracts. For example, degradation rates for red cabbage extract in a soft drink system could only be measured after 6 h at 80 ^{o}C.[152] However, it is generally accepted that acylated anthocyanins are more stable than non-acylated anthocyanins through thermal stress because of the protection of the flayvium cation through intramolecular copigmentation interactions[48]

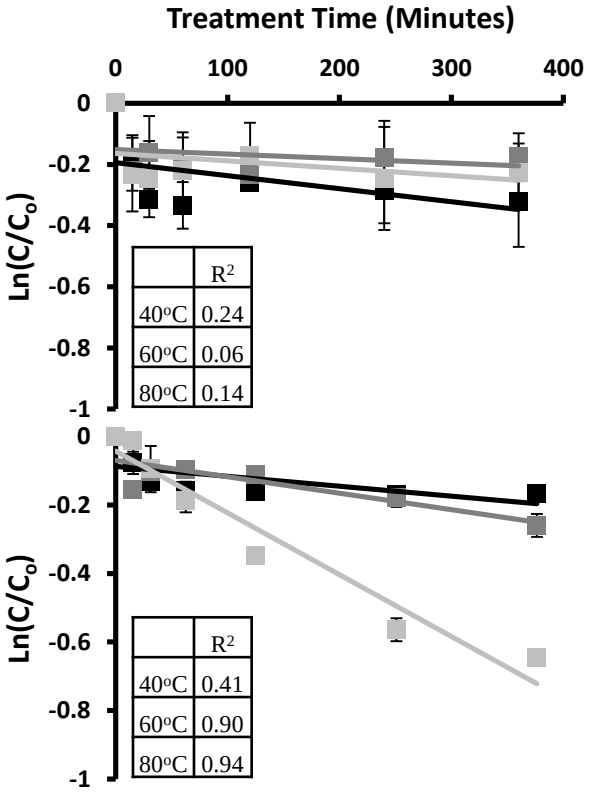


Figure 2.3 Total Monomeric Anthocyanin First Order Kinetic Plots PSP (Top) and Grape (Bottom) in VEW Model Beverages During Thermal Stress.
■, ■, and ■ correspond to 40, 60, and 80^{o}C treatments, respectively. "C" and "C_o" are the total monomeric anthocyanins concentration at a given time and the initial time, respectively.

2.3.6 Impact of Model Beverages on Anthocyanin Degradation Rates Through Photo Stress

With the exception of the preserved model beverage, high intensities of photo stress yielded greater total monomeric anthocyanin degradation rates **[Table 2.4]**. The decreased pH of the preserved base is likely to be responsible, in part, for this observation. The stability of anthocyanins has previously been shown to increase with decreasing pH.[141] Between 250 and 500 W/m^2 treatments, degradation rates significantly increased from 6.26x10^{-4} to 2.73x10^{-3} and 6.76x10^{-4} to 1.58x10^{-3} min^{-1} in 0 and 100 ppm AA model beverages, respectively. Additionally, total monomeric anthocyanin degradation rates significantly increased in the 500 ppm AA and VEW model beverages from 6.16x10^{-4} to 1.54x10^{-3} min^{-1} and 6.45x10^{-4} to 1.85x10^{-3} min^{-1} at 250 and 750 W/m^2, respectively. Furthermore, most anthocyanidin 3-O-glucosides followed the same significant differences as the total monomeric anthocyanin degradation rates providing further evidence of their similar susceptibility to photo stress **[Table 2.5]**.

Table 2.4 Total Monomeric Anthocyanin Degradation Rates and Activation Energies Under Photo Stress

Model Beverage	250 W/m^2(min^{-1})	500 W/m^2(min^{-1})	750 W/m^2(min^{-1})
0 ppm AA Hot Fill	6.26E-4 ± 8.6E-5^{1A}	2.73E-3 ± 9.7E-5^{2A}	3.59E-3 ± 2.1E-4^{3A}
100 ppm AA Hot Fill	6.76E-4 ± 2.7E-5^{1A}	1.58E-3 ± 1.5E-4^{2BC}	1.45E-3 ± 1.1E-4^{2BC}
500 ppm AA Hot Fill	6.16E-4 ± 4.9E-5^{1A}	1.16E-3 ± 1.0E-4^{12BD}	1.54E-3 ± 1.4E-4^{2BC}
VEW (Grape)	6.45E-4 ± 7.7E-5^{1A}	1.00E-3 ± 2.2E-5^{1BD}	1.85E-3 ± 1.0E-4^{2BD}
VEW (PSP)	1.29E-3 ± 3.3E-5^{1B}	2.02E-3 ± 2.6E-5^{2C}	2.40E-3 ± 1.9E-4^{2AD}
Preserved Model	7.33E-4 ± 1.2E-4^{1A}	9.20E-4 ± 5.4E-5^{1D}	1.16E-3 ± 8.2E-5^{1C}

Purple sweet potato extract, ascorbic acid and vitamin enriched water are abbreviated PSP, AA, and VEW, respectively. VEW contained either PSP or grape extract while all other model beverages contained grape extract. VEW model beverage (pH=3.60 ± 0.02) contained 0.025% AA and 0.044% vitamin water powdered premix. All hot filled model beverages (pH 3.60 ± 0.02)contained 0.60% citric acid and 1.00% sodium citrate, with various concentrations of AA indicated in the table. Preserved model beverage (pH=3.00 ± 0.02) contained 0.05% sodium benzoate, 0.01% potassium sorbate, 2% citric acid, and 0.60% sodium citrate. Different superscript numbers indicate significant differences (P<0.05) between light intensity within each model beverage. Different superscript letters indicate significant differences (P<0.05) between model beverages within each light intensity.

At 250 W/m^2, the total monomeric anthocyanin degradation rates in any of the model beverage bases were not significantly different. This may be attributed to the relatively low magnitude of photo stress and treatment time. Significant differences may be observed at lower light intensities at extended incubation times; however that was not investigated in the present study. In contrast, at 500 and 750 W/m^2, the addition of at least 100 ppm AA significantly decreased the total monomeric anthocyanin degradation rates **[Table 2.4]**. For example, the addition of 100 ppm AA caused the degradation rate to significantly decrease from 2.73x10^{-3} to 1.58x10^{-3} min^{-1} during the 500 W/m^2 photo stress treatment. Similarly, anthocyanidin 3-O-glucosides in the 0 ppm AA model beverage had significantly greater degradation rates than those in 100 and 500 ppm AA bases at 500 and 750 W/m^2, but not 250 W/m^2 **[Table 2.5]**. In contrast to thermal stress, increased concentrations of AA protected anthocyanins from photo stress. Typically, the presence of AA has been associated with the rapid degradation of anthocyanins in beverage systems. Our results clearly demonstrate that AA can have varying effects depending on whether thermal or photo stresses are the major concern.

Table 2.5 Grape Anthocyanin Degradation Rates Under Photo Stress

0 ppm AA Hot Fill			
	250 W/m^2(min^{-1})	500 W/m^2(min^{-1})	750 W/m^2(min^{-1})
Delphinidin 3-O-glucoside	1.51E-3 ± 2.3E-4$^{1A\alpha}$	9.12E-3 ± 1.2E-3$^{2A\alpha}$	1.02E-2 ± 2.1E-3$^{2A\alpha}$
Malvidin 3-O-glucoside	5.68E-4 ± 3.2E-5$^{1A\alpha}$	5.92E-3 ± 3.6E-4$^{2A\alpha}$	5.56E-3 ± 1.2E-4$^{2AB\alpha}$
Petunidin 3-O-glucoside	8.76E-4 ± 6.0E-5$^{1A\alpha}$	6.10E-3 ± 1.9E-4$^{2A\alpha}$	7.86E-3 ± 5.4E-4$^{2AB\alpha}$
Peonidin 3-O-glucoside	4.89E-4 ± 6.8E-6$^{1A\alpha}$	4.40E-3 ± 1.5E-5$^{2A\alpha}$	3.10E-3 ± 4.1E-4$^{2B\alpha}$
Cyanidin 3-O-glucoside	1.15E-3 ± 1.3E-4$^{1A\alpha\beta}$	7.41E-3 ± 6.7E-4$^{2A\alpha}$	6.76E-3 ± 1.5E-4$^{2AB\alpha}$
100 ppm AA Hot Fill			
	250 W/m^2(min^{-1})	500 W/m^2(min^{-1})	750 W/m^2(min^{-1})
Delphinidin 3-O-glucoside	5.40E-4 ± 6.5E-5$^{1A\alpha}$	2.00E-3 ± 3.2E-4$^{2A\beta}$	1.89E-3 ± 3.7E-4$^{2A\beta}$
Malvidin 3-O-glucoside	3.78E-4 ± 2.4E-5$^{1A\alpha}$	1.34E-3 ± 1.1E-4$^{2A\beta}$	1.01E-3 ± 2.4E-4$^{2A\beta}$

Table 2.5 Continued

Petunidin 3-O-glucoside	5.05E-4 ± 4.6E-5$^{1A\alpha}$	7.99E-4 ± 6.0E-5$^{1A\beta}$	1.63E-3 ± 3.1E-4$^{2A\beta\delta}$
Peonidin 3-O-glucoside	3.03E-4 ± 2.5E-5$^{1A\alpha}$	1.30E-3 ± 1.6E-4$^{2A\beta}$	1.24E-3 ± 3.0E-4$^{2A\beta\delta}$
Cyanidin 3-O-glucoside	7.75E-4 ± 4.5E-5$^{1A\alpha}$	2.16E-3 ± 8.0E-5$^{2A\beta\delta}$	2.20E-3 ± 1.1E-4$^{2A\beta\varepsilon}$
500 ppm AA Hot Fill			
	250 W/m^2(min^{-1})	500 W/m^2(min^{-1})	750 W/m^2(min^{-1})
Delphinidin 3-O-glucoside	1.00E-3 ± 7.5E-5$^{1A\alpha}$	1.51E-3 ± 2.6E-4$^{1AB\beta}$	1.66E-3 ± 1.9E-4$^{1AB\beta}$
Malvidin 3-O-glucoside	5.64E-4 ± 5.5E-5$^{1A\alpha}$	6.57E-4 ± 2.2E-4$^{1A\beta}$	1.15E-3 ± 7.6E-5$^{2A\beta}$
Petunidin 3-O-glucoside	8.07E-4 ± 4.9E-5$^{1A\alpha}$	1.13E-3 ± 1.4E-4$^{1AB\beta}$	1.71E-3 ± 1.2E-4$^{1AB\beta\delta}$
Peonidin 3-O-glucoside	5.69E-4 ± 2.7E-5$^{1A\alpha}$	7.28E-4 ± 1.3E-4$^{1AB\beta}$	1.41E-3 ± 1.1E-4$^{2AB\beta\delta}$
Cyanidin 3-O-glucoside	1.29E-3 ± 2.3E-5$^{1A\alpha\beta}$	1.74E-3 ± 1.2E-4$^{1B\beta}$	2.46E-3 ± 1.1E-4$^{1B\beta}$
Vitamin Enriched Water			
	250 W/m^2(min^{-1})	500 W/m^2(min^{-1})	750 W/m^2(min^{-1})
Delphinidin 3-O-glucoside	1.36E-3 ± 7.2E-4$^{1A\alpha}$	2.26E-3 ± 5.1E-4$^{1A\beta}$	3.45E-3 ± 5.6E-4$^{1AB\beta}$
Malvidin 3-O-glucoside	4.12E-4 ± 2.4E-4$^{1A\alpha}$	6.71E-4 ± 2.3E-4$^{1A\beta}$	1.48E-3 ± 2.9E-4$^{2A\beta}$
Petunidin 3-O-glucoside	1.27E-3 ± 3.1E-4$^{1A\alpha}$	1.72E-3 ± 2.5E-4$^{1A\beta}$	2.81E-3 ± 2.5E-4$^{2AB\beta}$
Peonidin 3-O-glucoside	1.05E-3 ± 4.6E-4$^{1A\alpha}$	1.08E-3 ± 1.5E-4$^{1A\beta}$	1.92E-3 ± 3.0E-4$^{1AB\alpha\beta}$
Cyanidin 3-O-glucoside	2.50E-3 ± 1.8E-4$^{1A\beta}$	3.44E-3 ± 1.1E-4$^{1A\delta}$	4.83E-3 ± 8.7E-5$^{2B\delta}$
Preserved Model			
	250 W/m^2(min^{-1})	500 W/m^2(min^{-1})	750 W/m^2(min^{-1})
Delphinidin 3-O-glucoside	1.21E-3 ± 1.6E-4$^{1A\alpha}$	9.12E-4 ± 7.1E-5$^{1A\beta}$	1.01E-3 ± 1.2E-4$^{1A\beta}$
Malvidin 3-O-glucoside	7.64E-4 ± 1.0E-4$^{1AB\alpha}$	3.19E-4 ± 4.5E-5$^{1AB\beta}$	3.33E-4 ± 2.1E-5$^{1B\beta}$
Petunidin 3-O-glucoside	1.16E-3 ± 1.2E-4$^{1AB\alpha}$	7.15E-4 ± 6.3E-5$^{1AB\beta}$	7.27E-4 ± 2.0E-5$^{1AB\delta}$
Peonidin 3-O-glucoside	5.80E-4 ± 7.1E-5$^{1B\alpha}$	2.52E-4 ± 1.1E-5$^{1B\beta}$	3.01E-4 ± 3.7E-5$^{1B\delta}$
Cyanidin 3-O-glucoside	9.55E-4 ± 6.2E-5$^{1AB\alpha}$	8.83E-4 ± 2.3E-5$^{1A\beta}$	1.03E-3 ± 6.3E-5$^{1A\varepsilon}$

VEW model beverage (pH=3.60 ± 0.02) contained 0.025% ascorbic acid (AA) and 0.044% vitamin water powdered premix. All hot filled model beverages (pH 3.60 ± 0.02)contained 0.60% citric acid and 1.00% sodium citrate, with various concentrations of AA indicated in the table. Preserved model beverage (pH=3.00 ± 0.02) contained 0.05% sodium benzoate, 0.01% potassium sorbate, 2% citric acid, and 0.60% sodium citrate. Different superscript numbers indicate significant differences ($P<0.05$) between light intensities within each model beverage and anthocyanin. Different superscript Roman letters indicate significant differences ($P<0.05$) between anthocyanins within each light intensities and model beverage. Different superscript Greek letters indicate significant differences ($P<0.05$) between model beverages within each light intensities and anthocyanin.

2.3.7 Grape and Purple Sweet Potato Anthocyanins Under Photo Stress

In contrast to thermal treatments, the total monomeric anthocyanin degradation rates of PSP and grape under photo stress both followed first order kinetics in the VEW

model beverage **[Figure 2.4]**. Correlation coefficients for PSP extract in the VEW model were 0.74, 0.90, and 0.93 for 250, 500, and 750 W/m^2 treatments, respectively. At 250 and 500 W/m^2, total monomeric anthocyanin degradation occurred significantly more rapidly with PSP than with grape **[Table 2.4]**. Previous studies report that acylated anthocyanins are more stable than non-acylated anthocyanins.[152, 164] For example, Hayashi et al. examined the impact of 8.8 W/m^2 (254 nm) on 19 anthocyanin rich extracts in a pH 3.16 buffer for 18 h.[164] Furthermore, they analyzed samples based on their absorbance at 525 nm, which is a measurement of the color of anthocyanins and may not necessarily be proportional to anthocyanin content, particularly with acylated

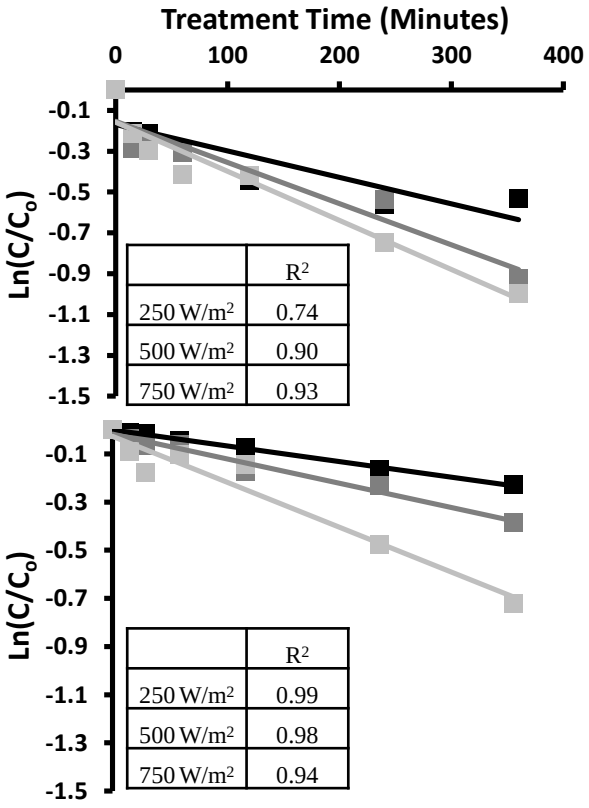


Figure 2.4 Total Monomeric Anthocyanin First Order Kinetic Plots for PSP (Top) and Grape (Bottom) in VEW Model Beverages During Photo Treatment.
■, ■, and ■ correspond to 250, 500, and 750 W/m^2 treatments, respectively. "C" and "C_o" are the total monomeric anthocyanins concentration at a given time and the initial time, respectively.

anthocyanins which undergo intramolecular copigmentation interactions. In the present study we used much greater light intensities at a range of wavelengths, different solutions, and analyzed the degradation of anthocyanins not based on the color, but rather the total monomeric anthocyanin concentration and individual anthocyanins by LC-MS. experiments.[9, 187] For example, peonidin-3-dicaffeoylsophoroside-5-glucoside was identified based on a parent ion of 1111 and fragments ions 949, 463, and 301 m/z corresponding to the removal of caffeic acid and/or glycosides **[Table 2.1]**. The degraded compounds were identified as diacylated peonidins, specifically peonidin-3-dicaffeoylsophoroside-5-glucoside, peonidin-3-caffeoyl-p-hydroxybenzoyl-sophoroside-5-glucoside, peonidin-3-caffeoyl-feruloylsophoroside-5-glucoside. The three forming compounds were tentatively identified as monoacylated peonidins, specifically peonidin-3-caffeoylsophoroside-5-glucoside, peonidin-3-p-hydroxybenzoyl-sophoroside-5-glucoside, and peonidin-3-feruloylsophoroside-5-glucoside. Thus, it is likely that the photo stress induced the removal of caffeic acid from the diacylated peonidins to yield the monoacylated peonidins. However, the concentration of peonidin-3-sophoroside-5-glucoside did not increase, indicating that monoacylated peonidins do not undergo a similar degradation mechanism. Furthermore, only one isomer of peonidin-3-caffeoylsophoroside-5-glucoside (949 m/z) was detected indicating that the photodegradative mechanism of peonidin-3-dicaffeoylsophoroside-5-glucoside involved the removal of caffeic acid from a specific position. Matsufuji et al. demonstrated that red radish extract containing seven diacylated pelargonidins will form seven new unidentified pigments when exposed to light and hypothesized that they were isomers.[162] However, they only obtained UV-Vis spectra and not mass spectra, thus they were unable

to test their hypothesis. From our findings, it is possible that some or all of these new pigments were monoacylated derivatives of the original seven diacylated pelargonidins \. However, unlike our findings, Matsufuji et al. also detected several of these new pigments through thermal treatments. Although this research is a step towards comprehending the chemistry of acylated anthocyanins, more research is required to fully understand the mechanisms involved.

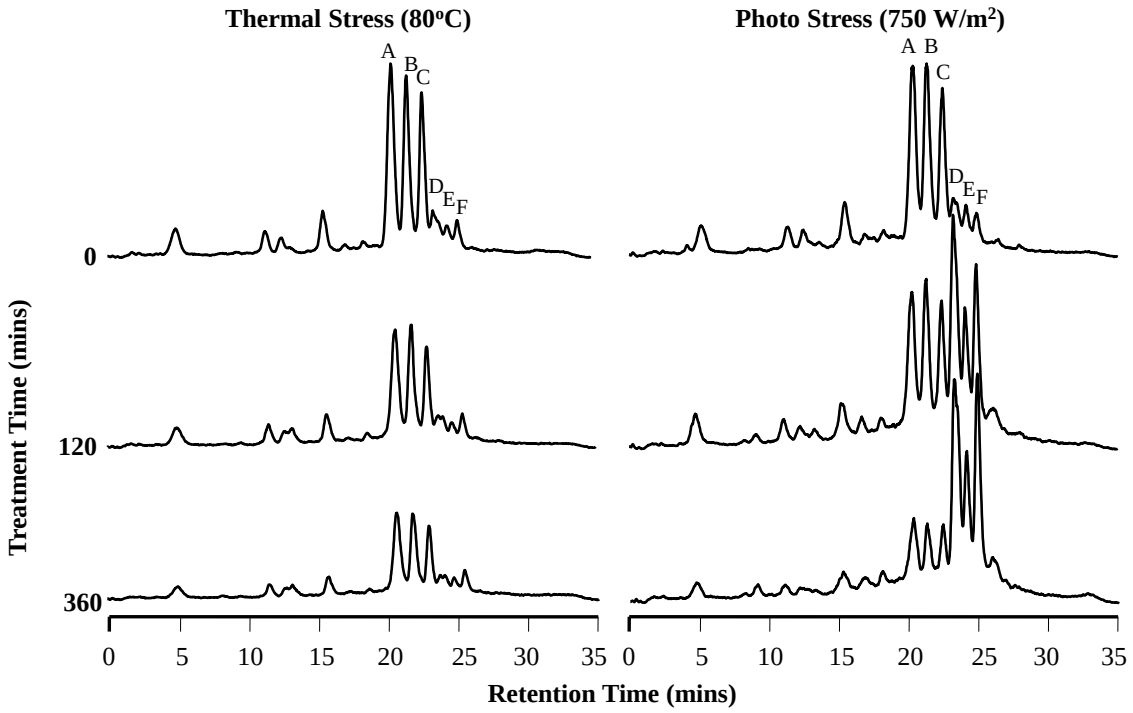


Figure 2.5 Normalized Extracted Ion Chromatograms for Peonidins (301 m/z) Through Thermal (Left) and Photo (Right) Stress at 0, 120, and 360 Min.
Labels A-F correspond to peonidin-3-dicaffeoylsophoroside-5-glucoside, -3-caffeoyl-p-hydroxybenzoyl-sophoroside-5-glucoside, -3-caffeoyl-feruloylsophoroside-5-glucoside, -3-caffeoylsophoroside-5-glucoside, -3-p-hydroxybenzoylsophoroside-5-glucoside, and -3-feruloylsophoroside-5-glucoside, respectively. D, E, and F increase in peak area during photo but not thermal stress.

2.3.8 Conclusions

In conclusion, the present study demonstrates the degradation kinetics of anthocyanins in the models of common RTD beverage formats through thermal and photo stress. Greater intensities of either stress resulted in increased degradation rates of both total monomeric anthocyanins and individual anthocyanidin 3-O-glucosides. Additionally, AA increased the degradation rates through thermal stress but decreased degradation rates through photo stress. Finally, photo stress resulted in the removal of a caffeic acid moiety from diacylated peonidins to form monoacylated analogues. These findings are likely to contribute to development of strategies for formulation of anthocyanins and anthocyanin rich ingredients in RTD beverages.

CHAPTER 3. ASSESSMENT OF PHYTOCHEMICAL CONTENT IN HUMAN MILK DURING DIFFERENT STAGES OF LACTATION

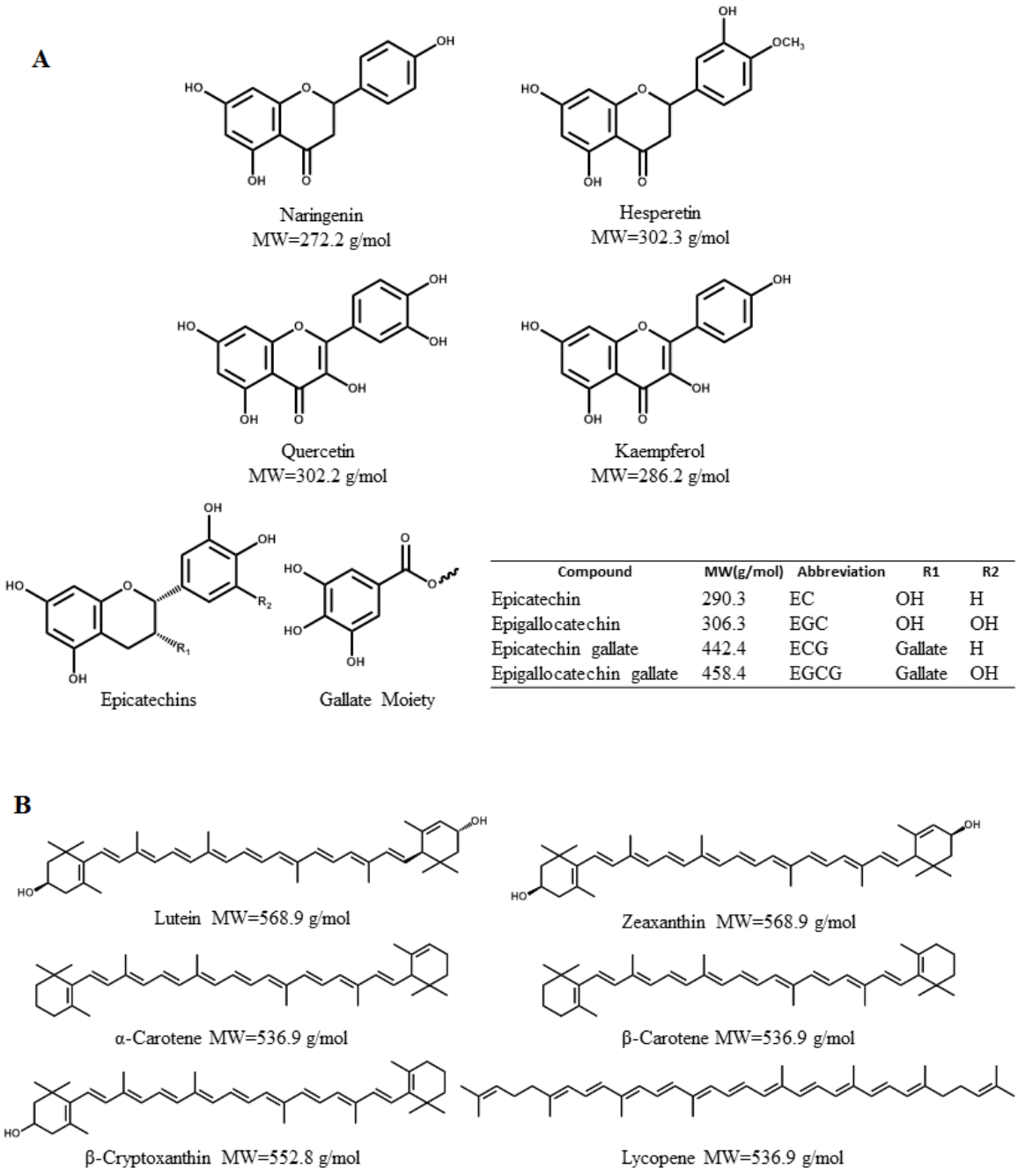
3.1 Introduction

Phytochemicals are secondary plant metabolites believed to impart health benefits including protection against oxidative stress and inflammation, and may reduce the risk of chronic and degenerative diseases such as cancer, obesity, diabetes and neurodegenerative disorders.[195-196] Two classes of phytochemicals, the flavonoids and carotenoids, have received significant attention in recent years due to their proposed nutritional and health promoting functions in humans. Carotenoids are a family of hydrophobic pigments abundant in algae and plants. While the provitamin A activity of β-carotene and β-cryptoxanthin is well documented, non-provitamin A carotenoids including lutein, zeaxanthin and lycopene have been increasingly studied for their biological activities including: antioxidant activity, cardiovascular protection, as well as eye health and skin health.[197-198] Flavonoids are a class of phytochemicals that broadly include flavonols, isoflavones, flavan-3-ols, flavanones. These polyphenols have demonstrated biological activities including antioxidant and anti-inflammatory activities consistent with promotion of vascular health, bone health, and cognitive function.[199-201] With the potential for a nutritional and health promoting role, interest in the content and variability of these phytochemicals from foods has grown significantly.

Adapted from Song, B. J.; Jouni, Z. E.; Ferruzzi, M. G., Assessment of phytochemical content in human milk during different stages of lactation. *Nutrition* **2013**, 29, 195-202, with permission.

For infants, human milk represents the primary and preferred source of nutrition. Previous studies have reported the composition of compounds in human milk that provide benefits beyond basic nutrition through stages of lactation.[202] Furthermore, carotenoid content of human milk has been the subject of many studies with the general conclusion that human milk content remains proportional to the mother's diet and correlates well to plasma carotenoid levels.[203-206] While these data support the notion that carotenoids are present in infant diets, little is known regarding the content of plant-derived flavonoids in human milk. Franke et al. [123-124, 207] characterized isoflavones in mothers consuming soy, however little information is available on natural levels of flavonoids in human milk including flavan-3-ols and flavonols commonly found in fruits and vegetables. More recently, Besle [208] identified flavonoids including quercetin, luteolin, and apigenin in milk of cows fed hay, maize and rye grass silage.

Considering the potential biological activity of plant derived carotenoids and flavonoids, additional insight into phytochemical profiles of human milk and their variation during lactation is required to better understand their potential exposure to and function in breast fed infants. The objective of the present study was to characterize the profiles of epicatechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate, naringenin, kaempferol, hesperetin, quercetin, lutein, zeaxanthin, α/β-cryptoxanthin, α/β-carotene, and lycopene **[Figure 3.1 A/B]** in human milk samples collected at different stages of lactation.



Compound	MW(g/mol)	Abbreviation	R1	R2
Epicatechin	290.3	EC	OH	H
Epigallocatechin	306.3	EGC	OH	OH
Epicatechin gallate	442.4	ECG	Gallate	H
Epigallocatechin gallate	458.4	EGCG	Gallate	OH

Figure 3.1 Structure of (A) Major Flavonoid and (B) Carotenoid Compounds Commonly Found in Fruits and Vegetables and Detected in Human Milk.

3.2 Material and Methods

3.2.1 Chemicals and Standards

L-ascorbic acid, Na_2-ethylenediaminetetraacetic acid (EDTA), pepsin (# P7000), NaOH, KOH, β-glucuronidase (#G0751), formic acid, 2,6-Di-tert-butyl-4-methylphenol (BHT), ethyl gallate, and β-apo-8-carotenal were purchased from Sigma Aldrich (St. Louis, MO). Solvents including ethyl acetate, methanol, isopropyl alcohol, acetone, petroleum ether, and HCl were purchased from Mallinckrodt Baker (Phillipsburg, NJ). Flavonoid standards including epicatechin, epigallocatechin, epicatechin-gallate, epigallocatechin-gallate, naringenin, kaempferol, hesperetin, and quercetin dihydrate standards were purchased from Sigma-Aldrich (St. Louis, MO). For carotenoids, zeaxanthin was purchased from Chromadex (Irvine, CA). β-cryptoxanthin, β-carotene, lycopene, and lutein standards were obtained from Sigma Aldrich (St. Louis, MO).

3.2.2 Human Milk Samples

Human milk samples (2 mL aliquots) were provided by the Cincinnati Children's Hospital Medical Center and is a subset of the Cincinnati cohort described by Woo et al. [209] Analysis of human milk samples was completed with approval from the Purdue University Institutional Review Board for Human Subject Research. Samples were collected from 17 women **[Table 3.1]** who delivered healthy term babies (≥37 weeks gestation) at a 1, 4, and 13 week post-partum time interval. The time points and sample size of 17 were based on the availability of samples from individual mothers at each time-point. This sample size is similar to other pilot investigation on human milk carotenoid composition.[203] As this was a preliminary investigation, diets were not

controlled or recorded and health records/clinical characteristics were not obtained. All women lived within 25 miles of the Cincinnati Children's Hospital Medical Center. On collection days, nurses visited the women between 10am and 1pm. The entire content of one breast was emptied using an electric pump. Milk samples were then aliquoted, coded, and frozen and stored at -70^{o}C. Researchers were blinded to the sample identity and time interval of each sample until the analysis was completed. All sample transfers and carotenoid extractions were completed under amber lights to minimize photo-oxidative reactions.

3.2.3 Flavonoid Extraction

Due to the absence of information on flavonoids content of human milk and the limited sample size available for analysis (2.0 mL), the decision was made to deconjugate potential metabolites (glucuronides and sulfate derivatives) to agylcones using β-glucuronidase/sulfatase treatment in an effort to simplify the separation and quantification of major flavonoids classes present in human milk. An aliquot of human milk (1 mL) was defatted with two 3 mL aliquot of hexane. Following removal of lipids, 50 µl of 2.7 mM L-ascorbic acid and 2.2 mM Na_2-EDTA in water was added followed by 6 mL of 40 mg/mL pepsin in 0.1 N HCl. Samples were incubated in a shaking water bath

Table 3.1 Demographic Data for Donor Mothers

	Mean[1]	**SD**
Age (years)	31.7	4.2
Weight (kg)	66.7	8.6
Length (cm)	164	5.0
# of Pregnancies	2.25	0.77
Birth Weight (g)	3490.4	342
Birth Height (cm)	51.0	1.6

[1] n=17

for 15 min at 37°C with mild agitation. Following incubation, the pH of the mixture was adjusted to 4.5 with 1.0 N NaOH and 3.85 kU of β-glucuronidase with sulfatase contaminant were added and samples were incubated for an additional 45 min at 37°C in a shaking water bath with mild agitation. Following enzymatic deconjugation, flavonoids were extracted three times with 3 mL aliquot of ethyl acetate. Ethyl acetate layers were collected, combined and dried under vacuum. Dried extracts were dissolved in 200 µl of mobile phase A prior to analysis.

3.2.4 Carotenoid Extraction

An aliquot (0.75 mL) of human milk sample was saponified with 0.3 mL of 30% methanolic KOH for 15 minutes at ambient temperature. Carotenoids were then extracted with 3:1 petroleum ether with 0.1% BHT:acetone a total of three times. Ether layers were collected combined and the solvent removed under vacuum. Dried extracts were resolubilized in 150 µl of 1:1 ethyl acetate and methanol prior to analysis.

3.2.5 Method Validation

The efficiencies of flavonoid and carotenoid extractions were evaluated by spiking freshly thawed human milk with ethyl gallate (in H_2O) and β-apo-8-carotenal (in ethanol) for final concentrations of 200 and 120 nmol/L respectively. Following spike addition samples were extracted as described previously. Average extraction efficiencies between triplicate experiments for the flavonoid and carotenoid extractions were 88.2 ± 2.3% and 75.4 ± 2.6%, respectively. Additionally, intraday and interday variations in extraction were evaluated for each analyte by a single operator who repeatedly extracted

and analyzed triplicate samples of a pooled human milk sample on three consecutive days **[Table 3.2]**. EGCG and EGC were not detected in the pooled sample, thus the coefficients of variation could not be calculated by this method. In order to approximate the coefficients of variation, green tea extract containing EGCG, EGC, and other flavan-3-ols was spiked into a separate aliquot of pooled human milk and extracted by the same operator in triplicate on three separate days. Finally, the limits of detection (LOD) for individual flavonoids and carotenoids were determined by serial dilutions prepared for each standard from a stock solutions of between 1.0 and 10 μmol/L. LOD was defined as a response three times the peak-to-peak noise level and results were expressed as the minimum detectable amount in nmoles per L of human milk **[Table 3.2]**.

Table 3.2 Coefficients of Variation and Limits of Detection for Flavonoid and Carotenoid Extractions.

	Intraday Coefficient of Variation (%)	**Interday Coefficient of Variation (%)**	**Limits of Detection (nmol/L of human milk)**
Epicatechin	4.3 ± 0.2	12.3 ± 1.0	13.5
Epicatechin Gallate	13.6 ± 4.5	16.6 ± 4.7	6.2
Epigallocatechin*	4.7 ± 0.6	4.0 ± 1.3	31.4
Epigallocatechin Gallate*	1.2 ± 0.7	9.1 ± 2.8	50.3
Naringenin	5.4 ± 4.9	7.1 ± 3.7	10.2
Kaempferol	9.5 ± 1.6	9.3 ± 5.0	0.4
Hesperetin	7.4 ± 1.7	9.3 ± 3.1	22.2
Quercetin	10.9 ± 3.0	13.7 ± 1.8	8.2
Lutein	6.5 ± 3.7	15.9 ± 3.3	0.3
Zeaxanthin	0.3 ± 0.2	15.1 ± 2.8	0.3
α-Cryptoxanthin	5.4 ± 2.8	12.1 ± 2.4	0.4
β-Cryptoxanthin	10.0 ± 8.3	13.1 ± 6.9	0.4
α-Carotene	18.8 ± 4.5	16.2 ± 3.8	0.3
β-Carotene	1.6 ± 0.8	2.3 ± 0.4	0.4
Lycopene	1.1 ± 0.9	3.4 ± 2.5	0.3

*EGC and EGCG were not detected in the pooled milk sample used for the calculation of coefficients of variation. Thus, intraday and interday coefficients of variation were estimated based on extraction of pooled human milk sample spiked with green tea extract.

3.2.6 Flavonoid Analysis via LC-MS

Flavonoid analysis was completed using a Waters 2695 Separations Module (Milford, MA) equipped with a Waters Xterra Reversed Phase C18 column (3.5 um 2.1 x 100 mm) (Milford, MA). Column and sample temperatures were set to 40 and 8^{o}C respectively. An elution gradient was used with a constant 0.30 mL/min flow rate and mobile phases A (0.4% formic acid in water), B (0.4% formic acid, 4% isopropyl alcohol in methanol), and C (methanol). Initial conditions were set at 98:2 (A/B) followed by a linear gradient to 55:42:3 (A/B/C) at 15 minutes, 20:80 (A/B) at 30 minutes, and finally a reset to 98:2 (A/B) at 35 minutes. Following elution, the column effluent was split 1:1 prior to introduction by electrospray ionization in negative ion mode (ESI-) into a Waters Micromass ZQ2000 MSD (Milford, MA). MSD conditions were set as follows: capillary voltage 3.5 kV, cone voltage 35 V, extractor voltage 3 V, RF lens 0.5 V, source and desolvation temperatures were set to 150 and 250^{o}C, respectively and desolvation and cone gas flow were set to 250 and 60 L/hr of nitrogen, respectively. The MSD was set to detect 2 groups of single ion responses (SIRs) at various time periods. Both groups of SIRs had dwell times of 0.2 seconds, inter-channel delays of 0.01 seconds, inter-scan delays of 0.01 seconds, and spans of 0.2. The first set of SIRs included 289, 305, 441, and 457 mass/charge signals for 0 to 18 minutes and targeted epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate, respectively. The second set of SIRs included 271, 285, 301, and 301 mass/charge signals for 16 to 35 minutes and targeted naringenin, kaempferol, hesperetin, and quercetin, respectively. Quantitation of each flavonoid was accomplished using calibration curves constructed from serial dilutions of authentic flavonoid standard stock

solutions. Standard solutions were subsequently injected on to the LC-MS to produce calibration curves for each compound at their corresponding mass/charge signals and elution times.

3.2.7 Carotenoid Analysis via LC-DAD

Carotenoid analysis was completed by LC-DAD using a gradient elution as described by Kean et al.[210] Separations were achieved on a Hewlett Packard 1090 equipped with a Waters YMC Carotenoid C30 column (2.0 x 150mm) (Milford, MA) and a guard column (2.0x50mm). Detection of carotenoids was accomplished using a Hewlett Packard 79880A diode array detector (DAD) scanning between 250-600nm at a 1.2nm resolution. Quantitation of lutein, zeaxanthin, α/β-cryptoxanthin and α/β-carotene was accomplished using the response at 450nm while lycopene quantitation was based on response at 470nm. Major carotenoids including all-E versions lutein, zeaxanthin, β-cryptoxanthin and α/β-carotene and lycopene were identified in human milk based on comparison of retention times and in-line electronic absorption spectra to those of authentic standards. Due to a lack of an authentic standard for α-cryptoxanthin, this carotenoid was tentatively identified based on elution order prior to β-cryptoxanthin and by in-line electronic absorption spectra.[211-212]

3.2.8 Lipid Analysis

The lipid content of human milk was determined using the creamatocrit method as described by Lucas et al.[213] An aliquot of freshly thawed human milk was drawn into 100µl capillary tube, sealed with clay, placed in a Clay Adams Readacrit Centrifuge

(Parsippany NJ) and centrifuged at 3000rpm for 15 minutes. The ratio of lipid length to column length was used to estimate the lipid percentage of the milk samples as previously described . Conversion of milk lipid percentage to grams of lipid per liter was accomplished using the equation lipid (g/L)=(creamatocrit (%)-0.59)/0.146.

3.2.9 Data Analysis

Flavonoid and carotenoid content of human milk is expressed as mean ± standard error of mean (SEM). Only samples where compounds were detected at quantifiable levels were used in calculation of mean and SEM. Data was analyzed using Statview version 5.0 (SAS Institute). Group differences were determined by analysis of variance and Fisher's PLSD post-hoc test (α =0.05).

3.3 Results

3.3.1 Flavonoid Content of Human Milk

Separation and detection of flavonoid aglycones from a representative sample of human milk can be seen in **Figure 3.2**. Single ion chromatograms (SIR) detected presence of epicatechin, epicatechin gallate, epigallocatechin gallate, naringenin, kaempferol, hesperetin, and quercetin as common flavonoids in human milk samples assayed in this study. The retention times of authentic standards and SIR signals (selected m/z) were used in combination to identify the flavonoids in samples.

Overall, the presence and concentration of flavonoids and in human milk samples encompassed a wide range **[Table 3.3]**. The limits of detection (LOD) for naringenin, epicatechin gallate, kaempferol and quercetin were determined to be LODs at 10.2, 6.2,

0.4, and 8.2nmol/L milk, respectively. Epicatechin, epigallocatechin gallate, epigallocatechin, and hesperetin had higher LODs at 13.5, 50.3, 31.4, and 22.2nmol/L milk, respectively. The difference in LODs likely influenced the number of samples individual flavonoids were detected in. For instance, quercetin and kaempferol were detected in all 51 samples while epigallocatechin gallate was only detected in 8 samples.

The flavonoid profile was compared in milk collected at 1, 4, and 13 week post-partum. Changes in the concentration of several flavonoids over time, such as epigallocatechin gallate, could not be fully examined because of their detection in only a

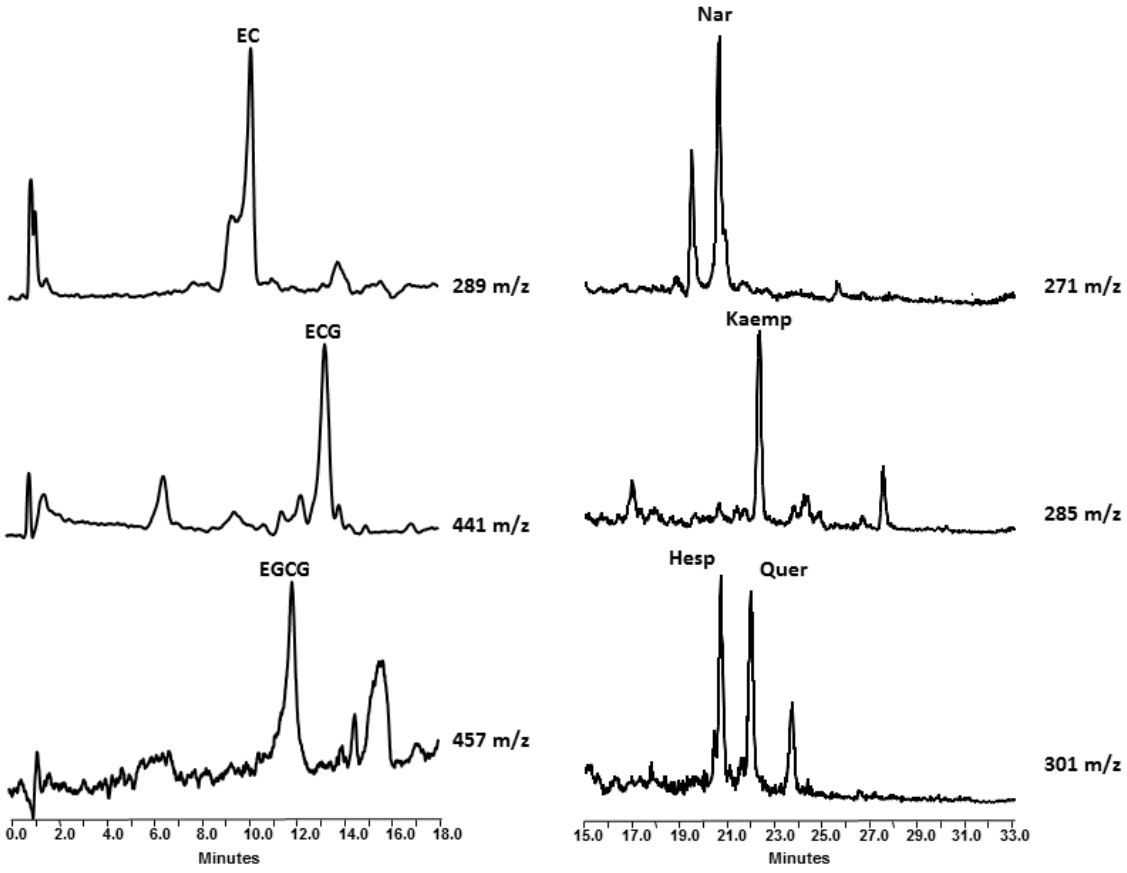


Figure 3.2 Representative LC-MS Selected Ion Response (SIR) Chromatograms Representing the Profile of Select Flavonoids Detected in Human Milk

limited number of samples **[Table 3.3]**. However, generally, minimal changes in flavonoids content over time were noted in human milk collected at different stages of lactation. Specifically, epicatechin, epicatechin gallate, hesperetin, quercetin, and naringenin content was not significantly different between weeks 1, 4 and 13 ($P>0.05$). However, kaempferol concentration was found to increase significantly with the week of lactation **[Table 3.3]**. Specifically, kaempferol concentration significantly increased between weeks 1 to 13 ($P<0.001$) and 4 to 13 ($P<0.005$). An increase was also noted between week 1 and 4 but this increase did not quite reach statistical significance ($P=0.06$).

3.3.2 Carotenoid Content of Human Milk

A representative chromatogram depicting the major carotenoids present in human milk can be seen in **Figure 3.3**. β-carotene and lutein were the most abundant carotenoids in these human milk samples **[Table 3.4]**. A sharp decrease in carotenoid content was noted as a function of lactation stage **[Table 3.4]**. β-carotene levels in human milk declined significantly ($P<0.05$) from 164.3 ± 25.2 to 88.0 ± 23.3nmol/L between weeks 1 and 13 respectively. Similar declines in carotenoid content were noted between week 1 and 4 for α-carotene (59.0 ± 13.5-19.2 ± 3.0nmol/L; $P<0.005$), α-cryptoxanthin (30.6 ± 4.8-16.8 ± 3.8nmol/L; $P<0.005$), β-cryptoxanthin (57.4 ± 10.7-27.5 ± 4.8nmol/L; $P<0.005$), lycopene (119.9 ± 18.9-68.0 ± 16.3nmol/L; $P<0.05$), lutein (121.2 ± 20.9-61.9 ± 10.9 nmol/L; $P<0.05$), and zeaxanthin (46.3 ± 5.4-22.8 ± 2.7nmol/L; $P<0.0001$) **[Table 3.4]**. Additional declines in carotenoid content were observed between week 4 and 13 but these were not found to be statistically significant.

Table 3.3 Flavonoid Concentration (nmol/L) in Human Milk Samples Collected from Free-Living Women at Week 1, 4 and 13 Post-Partum.

	Week 1				Week 4				Week 13			
Flavonoid	**n**[2]	**Range**[3]	**Mean**[3]	**SEM**[3]	**n**	**Range**	**Mean**	**SEM**	**n**	**Range**	**Mean**	**SEM**
Epicatechin	8	68.3 - 120.5	90.5[a]	7.1	5	63.7 - 828.5	249.2[a]	145.3	8	73.0 - 136.0	95.5[a]	7.9
Epigallocatechin	-	-	-	-	-	-	-	-	-	-	-	-
Epicatechin Gallate	17	55.7 - 609.3	189.5 [a]	36.7	11	62.2 - 645.6	236.6[a]	60.1	14	56.5 - 492.7	230.6[a]	39.7
Epigallocatechin Gallate	3	425.5 - 2364.7	1118.8[a]	624.3	-	-	-	-	5	215.1 - 1683.8	667.2[a]	267.3
Naringenin	17	82.9 - 542.6	252.1[a]	29.6	17	98.1 - 722.0	210.4[a]	39.3	15	64.1 - 447.0	196.6[a]	26.8
Kaempferol	17	7.8 - 34.0	15.7[a]	1.7	17	8.9 - 53.6	23.1[a]	2.9	17	17.7 - 71.4	34.8[b]	3.30
Hesperetin	12	107.1 - 1272.8	459.2[a]	123.6	13	79.9 - 1603.1	393.6[a]	119.5	11	74.8 - 704.7	352.0[a]	72.3
Quercetin	17	40.0 - 77.6	48.1[a]	2.2	17	33.1 - 108.6	59.8[a]	5.8	17	32.5 - 95.9	50.9[a]	3.4

[1] See Table 1 for demographic data
[2] Number of samples in which the specific flavonoid was positively detected. n=17 would indicate that the specific flavonoid was detected in all milk samples.
[3] Range, mean, and SEM calculated based on the number (n) of samples compound was detected in.
[4] Presence of different letter indicates a significant difference between flavonoid content in human milk of different lactation stage ($p<0.05$).

Carotenoid content of human milk also was expressed on the basis of its lipid content **[Table 3.5]**. Milk lipid content ranged between 22.20 and 89.93 g/L milk **[Figure 3.4]**. As before, β-carotene and lutein were highest in human milk and ranged between 0.5-9.7 and 0.9-6.8nmol/g of lipid in week one of lactation, respectively. Ranges of zeaxanthin, α-cryptoxanthin, β-cryptoxanthin, α-carotene and lycopene were found to be 0.5-2.1, 0.2-2.1, 0.4-5.8, 0.3-7.2, and 0.9-10.4nmol/g of lipid respectively in week one of lactation. When normalized for lipid content, β-carotene and lutein had the highest concentration throughout all weeks of lactation and also showed a significant decrease from weeks 1 to 13 ($P<0.05$). Similarly, significant declines were noted in α-carotene (1.6 ± 0.5-0.4 ± 0.0nmol/g lipid; $P<0.01$), α-cryptoxanthin (0.7 ± 0.1-0.3 ± 0.1nmol/g lipid; $P<0.01$), β-cryptoxanthin (1.4 ± 0.3-0.5 ± 0.1nmol/g lipid; $P<0.01$), lycopene (3.0 ± 0.6-1.2 ± 0.2nmol/g lipid; $P<0.01$), and zeaxanthin (1.1 ± 0.1-0.4 ± 0.0nmol/g lipid; $P<0.001$), between week 1 and 4. No significant difference was observed in carotenoid content between week 4 and 13 when expressed on milk lipid content **[Table 3.5]**.

3.4 Discussion

3.4.1 Flavonoids in Human Milk

While previously reported in bovine milk[208], to our knowledge, this is the first study to identify and quantify flavan-3-ols, flavonols, and flavanones in human milk. Samples included in this study were obtained from 17 free-living mothers at 1, 4 and 13 weeks post-partum. The selection of these time-points and samples were based on availability of matched samples for each mother at each time point of early, mid, and

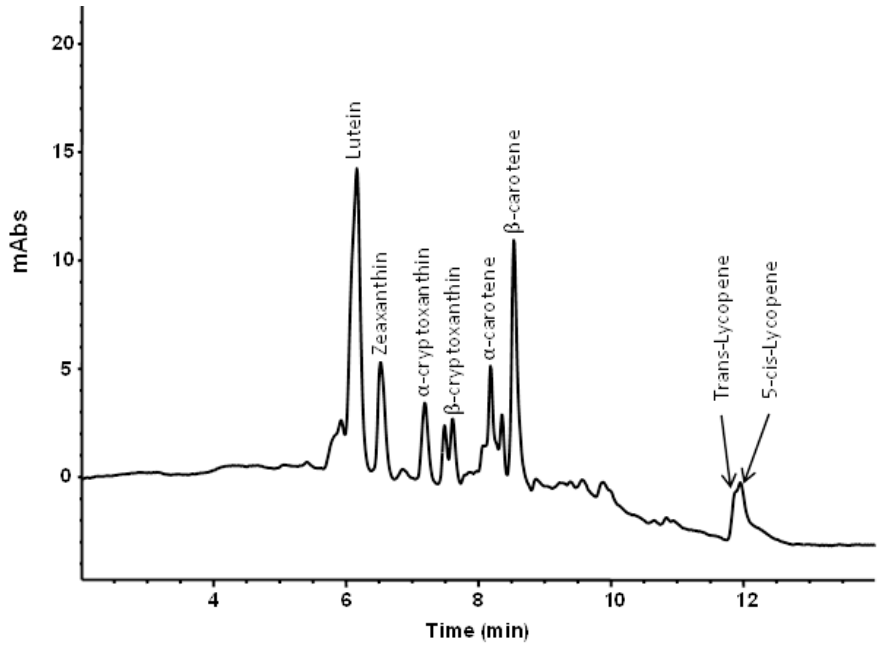


Figure 3.3 Representative LC-DAD Chromatograms Representing the Profile of Select Carotenoids Detected in Human Milk.
Signal at 450nm is depicted.

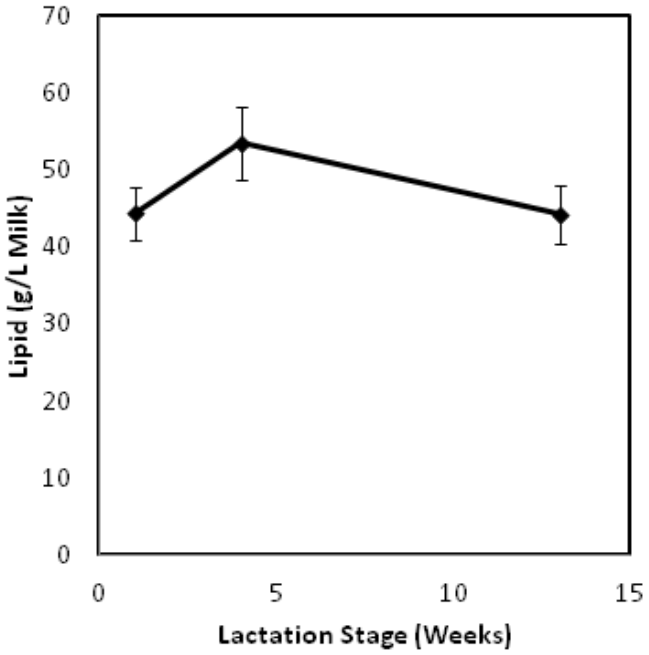


Figure 3.4 Change in milk lipid content between 1, 4 and 13 weeks of lactation.
Data represent mean ± SEM for n=17 individual samples for each time point.

Table 3.4 Carotenoid Concentration (nmol/L) in Human Milk from Free-Living Women at Week 1, 4 and 13 Post-Partum.

	Week 1				Week 4				Week 13			
Carotenoid	**n[2]**	**Range[3]**	**Mean[3]**	**SEM[3]**	**n**	**Range**	**Mean**	**SEM**	**n**	**Range**	**Mean**	**SEM**
Lutein	17	58.1 - 412.9	121.2[a]	20.9	15	16.8 - 193.4	61.9[b]	10.9	15	19.7 - 116.1	56.4[b]	6.8
Zeaxanthin	17	19.4 - 115.4	46.3[a]	5.4	15	11.9 - 52.6	22.8[b]	2.7	15	9.3 - 44.4	21.4[b]	2.5
α-Cryptoxanthin	16	11.9 - 72.6	30.6[a]	4.8	15	2.2 - 50.6	16.8[b]	3.8	16	2.3 - 33.1	13.5[b]	2.0
β-Cryptoxanthin	16	9.4 - 175.4	57.4[a]	10.7	17	2.1 - 70.7	27.5[b]	4.8	17	6.8 - 77.6	24.8[b]	4.4
α-Carotene	16	12.0 - 220.6	59.0[a]	13.5	14	7.3 - 46.5	19.2[b]	3.0	15	3.7 - 80.2	23.2[b]	4.8
β-Carotene	17	17.3 - 327.8	164.3[a]	25.2	16	10.7 - 377.4	104.4[a,b]	27.7	17	8.5 - 352.6	88.0[b]	23.3
Lycopene	17	30.5 - 317.5	119.9[a]	18.9	16	9.0 - 256.6	68.0[b]	16.3	17	19.2 - 103.0	49.5[b]	6.4

[1] See Table 1 for demographic data

[2] Number of samples in which the specific carotenoid was positively detected. n=17 would indicate that the specific flavonoid was detected in all milk samples.

[3] Range, mean, and SEM calculated based on the number (n) of samples compound was detected in.

[4] Presence of different letter indicates a significant difference between flavonoid content in human milk of different lactation stage ($p<0.05$).

Table 3.5 Carotenoid Concentration (nmol/g lipid) in Human Milk from Free-living Women at Week 1, 4 and 13 Post-Partum.

	Week 1				Week 4				Week 13			
Carotenoid	**n**[2]	**Range**[3]	**Mean**[3]	**SEM**[3]	**n**	**Range**	**Mean**	**SEM**	**n**	**Range**	**Mean**	**SEM**
Lutein	17	0.9 - 6.8	2.9[a]	0.4	15	0.5 - 2.3	1.1[b]	0.1	15	0.5 - 3.6	1.4[b]	0.2
Zeaxanthin	17	0.5 - 2.1	1.1[a]	0.1	15	0.3 - 0.6	0.4[b]	0.0	15	0.2 - 1.7	0.6[b]	0.1
α-Cryptoxanthin	16	0.2 - 2.1	0.7[a]	0.1	15	0.1 - 0.8	0.3[b]	0.1	16	0.1 - 0.8	0.3[b]	0.1
β-Cryptoxanthin	16	0.4 - 5.8	1.4[a]	0.3	17	0.1 - 1.0	0.5[b]	0.1	17	0.2 - 1.9	0.6[b]	0.1
α-Carotene	16	0.3 - 7.2	1.6[a]	0.5	14	0.2 - 0.7	0.4[b]	0.0	15	0.1 - 2.0	0.6[b]	0.1
β-Carotene	17	0.5 - 9.7	3.8[a]	0.7	16	0.2 - 8.2	2.1[a,b]	0.6	17	0.2 - 8.6	2.0[b]	0.6
Lycopene	17	0.9 - 10.4	3.0[a]	0.6	16	0.3 - 4.0	1.2[b]	0.2	17	0.5 - 2.5	1.1[b]	0.1

[1] See Table 1 for demographic data
[2] Number of samples in which the specific carotenoid was positively detected. n=17 would indicate that the specific flavonoid was detected in all milk samples.
[3] Range, mean, and SEM calculated based on the number (n) of samples compound was detected in.
[4] Presence of different letter indicates a significant difference between flavonoid content in human milk of different lactation stage ($p<0.05$).

later stage of lactation. Although dietary records were not collected from these women, these data suggests that dietary flavonoids are transferred from the blood into human milk at significant levels (nmol/L). Previous studies measuring flavonoids content of human milk have been limited to assessing one class of flavonoids, isoflavones, and specifically following milk levels in response to dietary intervention with soy.[207] In these studies, Franke et al. [124, 207] reported levels of daidzein and genistein in human milk at 80-110 and 30-50 nmol/L milk for following consumption of soy rich diets. These levels are similar to those found for flavan-3-ols, flavonols and flavanones in the present study **[Table 3.3]**, suggesting that these flavonoids are likely absorbed and transferred to human milk rapidly from the mother's diet and present in the diets of nursed infants. Furthermore, the generally consistent levels of flavonoids during the three time periods assayed in the present study suggest that accumulation of these compounds in human milk is not impacted by lactation stage. Considering the transient nature of flavonoids in biological fluids and tissues [214], human milk profiles may therefore be more reflective of daily dietary exposure and not accumulation of flavonoids in these fluids.

The qualitative flavonoids profile of human milk was also of particular interest. The eight flavonoids assessed in these samples were selected following a preliminary assessment of pooled milk samples. In addition, catechin, gallocatechin, catechin gallate, gallocatechin gallate, peonidin glucosides, malvidin glucosides, petunidin glucosides, cyanidin glucosides, and delphinidin glucosides were included in the preliminary assessment, however they were not detected and thus were excluded in the present study. Flavan-3-ols (epicatechin, epicatechin gallate, epigallocatechin, and epigallocatechin gallate) and well as flavonols (quercetin and kaempferol) and flavanones (naringenin and

hesperetin) were selected based on their predominance in these human milk samples and general prevalence in commonly consumed fruits, vegetables and beverages.[215-216] Of these polyphenols, naringenin and hesperetin were found in highest levels relative to favan-3-ols and flavonols. Flavanones are particularly abundant in citrus fruits and juices, such as oranges and grapefruits.[63] In contrast epigallocatechin gallate was found at high levels but in only 8 individuals. Typically, nursing mothers are advised to avoid flavan-3-ol containing foods/beverages such as tea because caffeine content. Considering that tea consumption may be restricted, but not completely avoided, the prevalence of decaffeinated tea products, and the high content of epigallocatechin gallate in tea [217], it is not surprising that a select group of individual samples had appreciable levels of epigallocatechin gallate present.

It is unclear if the flavonoids profile found in the present study is due to specific dietary exposure to specific flavonoids such as naringenin and hesperetin, or other factors including time of collection, kinetics of deposition, metabolism and stability of specific polyphenols in the milk matrix. In fact, the general instability of many flavonoids to near neutral pH conditions is well documented.[143] Additionally, the relative difference in antioxidant activity of the flavonoids may influence their presence in human milk. For instance, flavan-3-ols and flavonols have similar antioxidant activity, while flavanones have less antioxidant activity.[218] Thus, flavan-3-ols and flavonols in human milk are more likely susceptible to oxidative reactions, while flavanones may remain intact for longer periods of time. Therefore, naringenin and hesperetin may be present in high levels in the majority of mothers because of their higher oxidative stability in comparison to other flavonoids and merits further investigation.

Additionally, oxidation of flavonoids in milk based systems may be further impacted by association with specific macronutrients such as protein. Previous studies suggested that proteins in milk can interact with flavan-3-ols via proline-rich peptide segments [219] and that these interactions alter the oxidative stability of specific flavonoids.[220] It is plausible that flavonoids or their metabolites present in the human milk associate with by proline-rich segments of milk proteins increasing their stability to oxidative conditions in the milk. The extent to which differences in these associations and stability may alter the flavonoids profile of human milk and by extension the availability to the infant merit further study.

Furthermore, flavonoids are typically present in biological tissues and fluids as various metabolites, not their native aglycones or glycosides. While the chemical nature of the metabolites is specific to each flavonoid, typically most commonly flavonoids metabolites are found in plasma and tissues as glucuronides, methyl glucuronides, sulphates, and methyl sulphates.[221-222] Since flavonoids conjugation to these metabolites may occur in both the small intestines and the liver, it is likely that the flavonoid metabolites are available to be transferred into the milk prior to hepatic metabolism. While further research is needed to determine the presence and relative concentration of specific flavonoid metabolites in human milk, the consistent presence of specific flavonoids in human milk samples collected over a 13 week period from free living mothers suggest that these compounds are being effectively transferred into human milk and made available for absorption by a nursing infant.

3.4.2 Carotenoid Content of Human Milk

Although lipid and carotenoid content in human milk has previously been reported, few studies focused on their concentration from 1 to 13 weeks postpartum. Generally, the lipid content and variability was similar to that previously shown for human milk at later stages of lactation.[223] In addition, the fluctuation of milk lipid content between 1 and 13 weeks post-partum was similar to what was observed by Michaelsen et al.[224] Schweigert et al. and Jewell et al. examined the milk carotenoid concentrations between 1 and 41 days postpartum.[203, 205] Studies by Canfield et al. and Lietz et al. examined a wider range of time then the previously mentioned studies, however their focus was specifically on β-carotene and red palm oil supplements, respectively.[225-226] In the present study, human milk carotenoid content was found to decrease over lactation stage but generally had less variability in content relative to flavonoids. This is consistent with previous studies that examined both classes of phytochemicals in plasma.[227]

α-Carotene, α/β-cryptoxanthin, lycopene, lutein, and zeaxanthin significantly decreased with increasing stages of breastfeeding, specifically between 1 to 4 and 1 to 13, but not 4 to 13 weeks postpartum. These data suggest that carotenoid content in human milk decreases during early stages of lactation but then only modestly decreases during the next 9 weeks. These data are similar to that obtained by Schweigert et al. in 2004 during a shorter period. Schweigert et al. reported significant decreases in carotenoid concentration in human milk between 4 and 19 day post partum intervals.[205] The significantly greater carotenoid concentration in human milk during the first week postpartum is likely to be caused partially by the storage of milk before the infant was

breastfed. Prior to the first week postpartum, human milk was not being eliminated from the breasts and carotenoids may have accumulated over a period of time. Once the infant was breastfed, the milk repositories are continuously emptied, thus carotenoids were not accumulated for a longer period of time.

Lutein, β-carotene, and total lycopene had the highest concentration throughout every week in this study. These results are consistent with carotenoid content of typical western diets. Canfield et al. examined the carotenoid concentrations in breast milk from 9 nations, including the United States of America and the United Kingdom.[204] Both countries had similar qualitative profile where lutein, β-carotene, and total lycopene were the most abundant. Similarly, Jewell et al. examined human milk samples from mothers in Ireland and found that lutein and β-carotene had the highest concentrations, but total lycopene was not measured.[203]

3.5 Conclusion

Flavonoids and carotenoids were identified and quantified in human milk during 1, 4, and 13 week postpartum for 17 mothers. To our knowledge, this was the first study to report flavonoids in human milk. Further research is required to better understand the role these phytochemicals may play in infant health.

CHAPTER 4. MULTINATIONAL SURVEY OF FLAVONOIDS IN HUMAN MILK

4.1 Introduction

In our preliminary evaluation of human milk, flavonoids were detected in samples collected from 17 free-living mothers at 1, 4, and 13 weeks postpartum.[228] Epicatechin (63.7-828.5nmol/L), epicatechin gallate (55.7-645.6nmol/L), epigallocatechin gallate (215.1-2364.7nmol/L), naringenin (64.1-722.0nmol/L), kaempferol (7.8-71.4nmol/L), hesperetin (74.8-1603.1nmol/L), and quercetin (32.5-108.6nmol/L) were all present in human milk samples with high inter/intra-individual variability.[228] The study was designed to be a feasibility assessment and preliminary scan of flavonoids in human milk of a free living small cohort of donors in the United States. While establishing the first evidence of flavonoid in human milk of free-living mothers, the effect of diet and regional differences in milk profiles is unknown. Furthermore, due to limited sample volumes in the pilot study, the presence and content of specific flavonoid metabolites remains unknown.

The dietary exposure to flavonoids directly impacts their presence and the presence of their metabolites in plasma and other biological tissues.[229-230] Although flavonoid consumption greatly varies between individuals and age in free-living populations, certain geographical locations are associated with greater exposure to overall flavonoids and specific classes of flavonoids.[16-19] For instance, daily average intake of

multiple flavonoids was estimated to be 23-46, 24, 73, 63, and 20-34 mg/day for Denmark, Finland, Holland, Japan, and The United States, respectively.[1] Furthermore, with the exception of anthocyains, most flavonoids are generally not present in tissues as their aglycones or glycosides found in typical fruits and vegetables.[100] In contrast, consumed flavonoids are generally subjected to multiple phase-II conjugation enzymes including UDP-glucuronosyltransferases (UGT), catechol-O-methyltransferases (COMT), and phenolsulfosyltransferases (SULT), responsible for these conjugation mechanisms.[95, 100, 104-105] Thus, flavonoids in biological tissues and fluids, including human milk, are likely to be present as methylated, glucuronidated, and sulfonated molecules.[104] To date, information of geographic differences and presence of metabolites in human milk is absent. In the present study, flavonoid profiles in human milk samples collected from 60 donors in The United States of America, China, and Mexico through 4 lactation stages was assessed. Additionally, pooled samples were assayed for individual flavonoid metabolites and assay for presence of broader classes of flavonoids. A deeper understanding of flavonoid deposition in human milk may provide further insight to their biological relevance in women and their nursing infants.

4.2 Materials and Methods

4.2.1 Sample Information

Collection and shipment of human milk samples was coordinated by Mead Johnson Nutrition (Evansville, IN) and the Cincinnati Children's Hospital Medical Center. Samples were shipped to Purdue on dry ice and stored at -80°C. Longitudinal samples were collected at 2, 4, 13, and 26 weeks postpartum from 60 donors, 20 from

each of three nations: China, Mexico, and USA (Cincinnati). Each sample was received as two separate 2 mL aliquots in (480 vials in all). One vial from each sample was assigned to either carotenoid (to be reported separately) or flavonoid analysis to minimize freeze-thaw cycling. Sample identification was blinded except for country of origin, and vials were assigned to a random order for analysis.

4.2.2 Materials

Epicatechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate, naringenin, kaempferol, hesperetin, quercetin dehydrate, L-ascorbic acid, Na_2-ethylenediaminetetraacetic acid, pepsin (P7000), β-glucuronidase (G0751), formic acid, ethyl gallate, and HPLC-grade denatured ethanol were from Sigma-Aldrich (St. Louis, MO). Solvents including ethyl acetate, methanol, hexanes and HCl were purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA).

4.2.3 Flavonoid Aglycone Extraction

Flavonoids were deconjugated and extracted as described by Song et al. with minor modifications.[228] Briefly, an internal standard of 0.5 mL of 4.07 μg/L ethyl gallate was transferred into 1 mL milk to serve as an internal standard. Milk was then defatted with hexane. L-ascorbic acid and Na_2-ethylenediaminetetraacetic acid in water was added followed by pepsin in 0.1 N HCl. Samples were incubated in a shaking water bath for 15 min at 37°C with mild agitation. After incubation, the pH of the mixture was adjusted to 4.5 with 1.0 N NaOH, β-glucuronidase with sulfatase contaminant was added, and the samples were incubated for an additional 45 min at 37°C in a shaking water bath

with mild agitation. After enzymatic deconjugation, flavonoids were extracted with ethyl acetate. The ethyl acetate layers were collected, combined, and dried under vacuum. The dried extracts were dissolved in mobile phase A before analysis.

4.2.4 Flavonoid Aglycone Analysis

Flavonoid analysis was completed using a Hewlett Packard 1100 Series HPLC (Miami, Fl) coupled to an LCT Premier time-of-flight mass spectrometer (TOF-MS) from Waters (Milford, MA). Extracted ion chromatograms for aglycones in human milk are shown in [**Figure 4.1**]. The column compartment was thermostated at 40°C. A Waters Xterra Reversed Phase C18column (3.5 mm, 2.1x100 mm) was used with a flow rate of 0.30 mL/min flow rate and gradient elution of mobile phases A (0.4% formic acid in water), B (0.4% formic acid, 4% isopropyl alcohol in methanol), and C (methanol). The initial conditions were set at 98:2 (A/B) followed by a linear gradient to 55:42:3 (A/B/C)

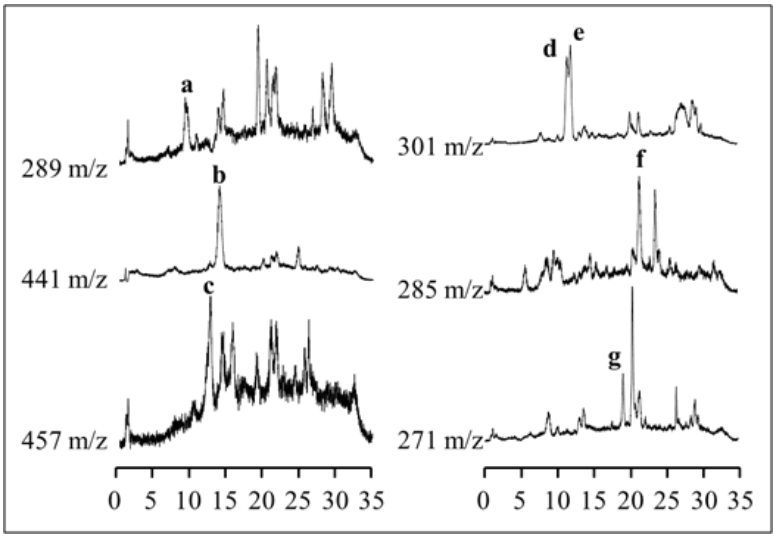


Figure 4.1 Extracted Ion Chromatograms of Flavonoid Aglycones from Human Milk Samples.
Identification: (a) epicatechin, (b) epicatechin gallate, (c) epigallocatechin gallate, (d) quercetin, (e) hesperetin, (f) kaempferol, (g) naringenin.

at 15 min,20:80 (A/B) at 30 min, and a reset to 98:2 (A/B) at 35 min. After elution, the column effluent was split 1:1 to the TOF-MS and diode-array detector. The TOF-MS was equipped with electrospray ionization (ESI) using nitrogen carrier gas. Other conditions as follows: capillary voltage 3.5 kV, cone voltage 35 V, desolvation temp 250°C, source temp 150°C, desolvation gas 250 L/hr, cone gas 60 L/h of nitrogen. TOF scan 50 to 700 m/z in negative ion V mode (single reflectron), with scan duration 1 sec and interscan delay of 0.1 sec. Each flavonoid was quantified using calibration curves constructed from serial dilutions of authentic flavonoid standard stock solutions. Standard solutions were subsequently injected on to the liquid chromatography–mass spectrometry to produce the calibration curves for each compound at its corresponding m/z signals and elution times. The limit of detection was calculated to be 0.27, 0.50, 0.68, 0.26, 0.56, 0.07, and 0.21 nmol/L in milk samples for epicatechin, epicatechin gallate, epigallocatechin gallate, quercetin, kaempferol, naringenin, and hesperetin, respectively.

4.2.5 Flavonoid Metabolite Extraction

Samples were pooled to increase sample volume and thus enhance analytical sensitivity for flavonoid metabolites. For each country x lactation stage group (20 donors each), remaining sample aliquots were pooled into 2 separate pools (10 donors each), with 1 mL from each donor. Flavonoid metabolites were extracted with a similar method as flavonoid aglycones described above, except for proportionally increasing solvent volumes, omitting β-glucuronidase/sulfatase hydrolysis of conjugates, and finally, by application of solid phase extraction with Oasis HLB cartridges (Waters). Briefly, cartridges were prepared by rinsing with methanol and deionized water. Milk samples

were loaded onto the cartridge, washed with 1.5 M formic acid in water, and 95:5 water:methanol, sequentially. Finally, flavonoid metabolites were eluted from the cartridge with 0.1% formic acid in methanol, dried under nitrogen, and reconstituted in 2% formic acid in water.

4.2.6 Flavonoid Metabolite Analysis

Metabolites were analyzed by HPLC-MS in the same manner as aglycones, monitoring corresponding ions. Due to the lack of authentic individual flavonoid metabolite standards, archived rodent plasma samples (from previous studies in our group[231-233]) containing several of these metabolites were used to compare chromatograms and mass spectra collected in-line in order to tentatively identify observed ions **[Appendix A]**. Metabolites that were not in the rodent plasma were tentatively identified based on relative elution time and collected in-line mass spectra. Concentrations were estimated based on calibration curves constructed from the corresponding aglycones of the metabolites. The limit of detection was calculated to be 0.05, 0.10, 0.14, 0.05, 0.11, 0.01, and 0.04 nmol/L in pooled milk samples for epicatechin, epicatechin gallate, epigallocatechin gallate, quercetin, kaempferol, naringenin, and hesperetin, respectively.

4.2.7 Anthocyanin Extraction

In our first preliminary screen, ten samples from each cohort (thirty total) were randomly selected, extracted, and analyzed. In a second anthocyanin screen, thirty samples with the greatest polyphenol concentrations were selected in order to enhance the

probability of detecting anthocyanins. All samples were prepared by solid phase extraction on Oasis HLB cartridges (Waters). Briefly, cartridges were prepared by rinsing with methanol and deionized water. Milk samples were loaded onto the cartridge, washed with 2% formic acid in water. Finally, flavonoid metabolites were eluted from the cartridge with 2% formic acid in methanol, dried under nitrogen, and reconstituted in 2% formic acid in water.

4.2.8 Anthocyanin Analysis

Analyses of anthocyanins and anthocyanidin glucuronides were performed on an Agilent 6400 Triple Quadrupole LC-MS/MS equipped with an electron spray ionization (ESI) source under multiple reaction monitoring modes (MRM). A Waters XTerra RP-C18 column (2.1 mm x 100 mm, 3.5 μm particle size) was used with binary mobile phases: (A) 2% aqueous formic acid (v/v) and (B) 0.1% formic acid in acetonitrile (v/v). The column was heated to 35℃ and the system flow rate was 0.3 mL/min. The binary gradient to elute anthocyanins and anthocyanidin glucuronides was 5% B at 0 min, 10% B at 10 min, 25% B at 30 min, 5% B at 31 min and continue on 5% B to 35 min. Fragmentor voltage was set at 135V and collision energy was 17eV for all compounds. ESI source conditions: gas temperature was 350℃, drying gas flow was 11 l/min, nebulizer was 30 psi, sheath gas temp was 350℃, sheath gas flow was 11 L/min, capillary voltage was 3500 V and nozzle voltage was 1000 V. MRM transitions are listed in **Table 4.1**. Retention times for MRMs were compared between milk samples and rodent plasma samples using the same method that were known to contain anthocyanins and their

metabolites. The limit of detection for malvidin 3-O-glucoside was calculated to be 0.04 nmol/L milk.

4.2.9 Statistical Analysis

Data were analyzed using SAS 9.3 (SAS Institute, Cary, NC). Due to the skewness and non-normality of phytochemical concentrations, transformations were identified using the Box-Cox procedure [**Table 4.2**]. Particular observations below the limit of detection were reported as not detected (instead of including in analysis as concentration = 0). Analysis of variance (ANOVA) for each transformed concentration was completed using a factorial model of country (fixed categorical variable), week (continuous variable, i.e. linear regression), donor nested within country (blocking variable to increase power), and country by week interaction. P values represent type III sum of squares (with all other variables in the model). Superscripts denote significant differences between countries (α=0.05) with all other variables in the model, with superscript a (^{a}) denoting the country with the highest concentration. Box plots represent median, interquartile range (IQR), 25th percentile – 1.5 x IQR (or minimum, whichever is closer to the median), and 75th percentile + 1.5 x IQR (or maximum, whichever is closer to the median). Correlations between countries and frequency of a specific flavonoid detection was investigated using a chi-square test. 95% confidence intervals were used to detect deviations from even distribution between the three countries.

Table 4.1 MRM Transitions of Anthocyanins and Anthocyanidin Glucuronides.

Anthocyanin	MRM transition
malvidin glucoside	493.1 → 331.1
petunidin glucoside	479.1 → 317.1
delphinidin glucoside	465.1 → 303.1
peonidin glucoside	463.1 → 301.1
cyanidin glucoside	449.1 → 287.1
malvidin glucuronide	507.1 → 331.1
petunidin glucuronide	493.1 → 317.1
delphinidin glucuronide	479.1 → 303.1
peonidin glucuronide	477.1 → 301.1
cyanidin glucuronide	463.1 → 287.1

Table 4.2 ANOVA for Flavonoid Aglycone Content on a Volume Basis.

volume basis	*p-value of variable*				
	transformation	**country**	**week**	**donor**	**country*week**
epicatechin	y^(0.5)	0.25	0.52	0.02	0.42
epicatechin gallate	ln(y)	0.01	0.08	0.09	0.3
epigallocatechin gallate	y^(-0.25)	0.16	0.73	0.77	0.064
quercetin	y^(0.25)	0.47	0.085	0.47	0.3
kaempferol	y^(-1)	0.55	0.35	0.23	0.18
naringenin	y^(0.25)	0.21	0.11	0.30	0.005
hesperitin	y^(-0.25)	0.49	0.59	0.37	0.015
total aglycones	**ln(y)**	**0.18**	**0.11**	**0.0014**	**0.02**

4.3 Results and Discussion

4.3.1 Flavonoid Aglycones

Similar to our previous investigation, epicatechin, epicatechin gallate, epigallocatechin gallate, quercetin, naringenin, kaempferol, and hesperetin were detected in human milk samples after enzymatic hydrolysis of their metabolites [**Table 4.3**]. Statistical measures were calculated for samples where flavonoids were above the limit of detection. Lactation stage was not correlated to the concentration of any flavonoids. Additionally, flavonoid content was not associated with donor ($p>0.05$ for all except epicatechin). The transient nature of flavonoids in biological fluids and tissues as well as variations in consumption likely in free living individuals between weeks may contribute to these observations.

Milk samples from China had significantly greater total flavonoid aglycone content in comparison to the American cohort. However, the Mexican cohort was not significantly different from either [**Figure 4.2**]. Epicatechin, epigallocatechin gallate, quercetin, naringenin, and hesperidin concentrations were not significantly different between any country. However, the Mexican cohort had significantly higher concentrations of epicatechin gallate than the American cohort, but not the Chinese cohort. Furthermore, the Chinese cohort had significantly higher concentrations of kaempferol than the American cohort, but not the Mexican cohort [**Figure 4.3**]. Differences in flavonoid content between cohorts may be related to differences in dietary exposure between cultures. While dietary records were not available from these subjects, total flavonoid consumption for Mexico, USA, and China populations has been previously estimated at 158.0, 189.4, and 165.8 in three different studies.[16, 234-235]

However, it should be noted that these studies vary in phytochemical screening, sample selection, and time period. For instance, flavan-3-ol intake was oddly not factored into the total flavonoid intake calculation for China, despite it being considered one of the most abundant classes of flavonoids in tea, apples and other fruits and one of the most abundant in the diet. [235] Considering that tea is a widely consumed beverage in China and both monomeric and polymeric flavan-3-ols are major contributors to dietary exposure to flavonoids, 165.8 mg/day is likely to be an underestimate. Furthermore, the significant differences in kaempferol and epicatechin gallate does not correlate with the data in the flavonoid intake studies. This is likely caused, in part, by the difference in data reporting and representation between intake and concentrations in biological fluids. Flavonoids are transient in biological fluids and their presence greatly varies with time from consumption and generally follows a dose dependent response.[56, 103] In contrast, flavonoid intake studies are averages of many individuals that meet specific criteria over a longer period of time. Thus, flavonoid deposition in human milk may not correspond with flavonoid intake studies. It should be noted that these statistical analyses are on observations with detectable levels, and that the frequency of flavonoid detection did vary between cohorts. For instance, although there was no significant difference in epigallocatechin gallate concentration between countries, its presence was more common in samples from China than those from either Mexico or the USA. However, these trends did not reach the level of significance ($\alpha=0.05$) and is likely a result of relatively smaller sample size, resulting in low statistical power. Currently, large quantities of human milk samples from multiple cohorts are difficult to obtain for flavonoid profiling because of

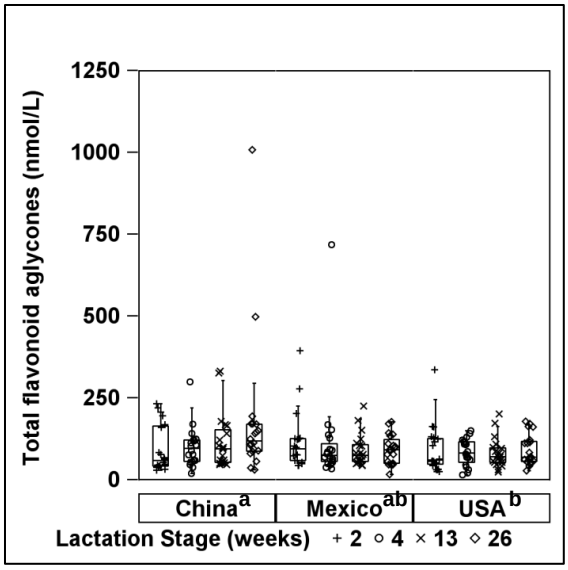


Figure 4.2 Total Flavonoid Aglycones of Human Milk Samples.
Determined by the summation of flavonoid concentrations described in **Table 5.2**.

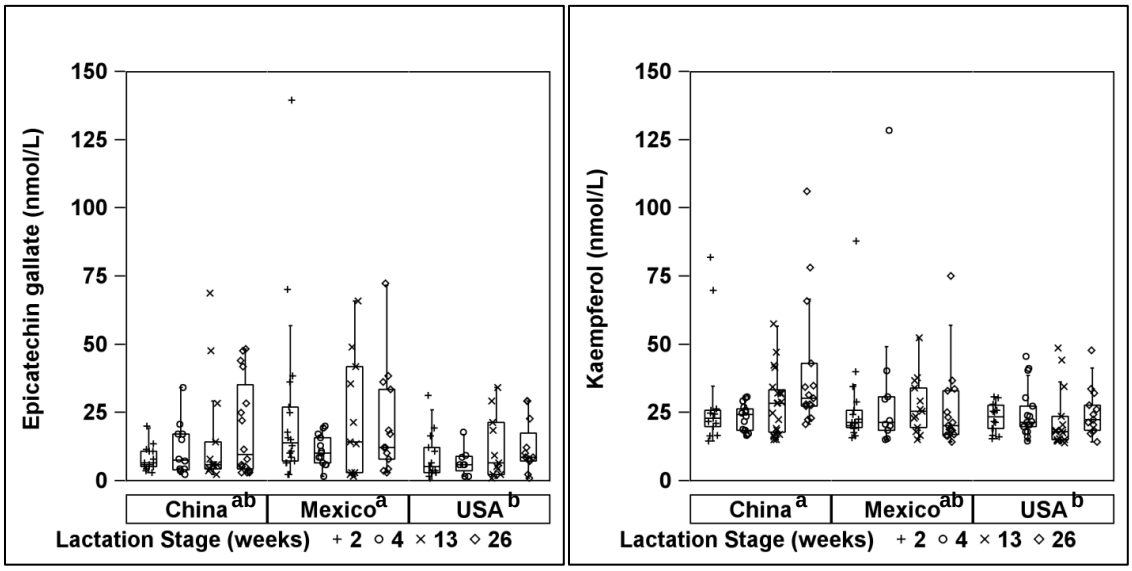


Figure 4.3 Epicatechin Gallate and Kaempferol Concentrations in Human Milk Samples.
Different superscript letters indicate significant differences between countries.

Table 4.3 Flavonoid Aglycone Content (nmol/L) by Country.

nmol/L	China			USA			Mexico			Overall		
	mean ± STDEV	(min, max)	detection frequency	mean ± STDEV	(min, max)	detection frequency	mean ± STDEV	(min, max)	detection frequency	mean ± STDEV	(min, max)	detection frequency
epicatechin	14.0 ± 10.2	(0.6, 41.0)	72/80, 90.0%	15.1 ± 10.1	(1.8, 40.2)	71/80, 88.8%	15.8 ± 10.2	(0.6, 44.7)	67/80, 83.8%	14.9 ± 10.1	(0.6, 44.7)	210/240, 87.5%
epicatechin gallate	13.9 ± 15.0	(2.2, 68.7)	54/80, 67.5%	10.0 ± 9.2	(0.1, 34.0)	46/80, 57.5%	20.2 ± 23.6	(1.5, 139.5)	55/80, 68.8%	15.0 ± 17.8	(0.1, 139.5)	155/240, 64.6%
epigallocatechin gallate	21.6 ± 23.7	(2.5, 111.7)	74/80, 92.5%	21.0 ± 23.0	(2.5, 115.4)	64/80, 80.0%	22.4 ± 23.6	(3.3, 116.9)	59/80, 73.8%	21.6 ± 23.4	(2.5, 116.9)	197/240, 82.1%
quercetin	20.1 ± 15.1	(1.2, 76.9)	78/80, 97.5%	17.7 ± 14.7	(2.6, 100.0)	76/80, 95.0%	18.2 ± 11.8	(3.6, 67.0)	77/80, 96.3%	18.7 ± 14.0	(1.2, 100.0)	231/240, 96.3%
kaempferol	30.0 ± 17.3	(14.4, 106.1)	64/80, 80.0%	23.7 ± 8.8	(13.8, 48.6)	59/80, 73.8%	28.0 ± 19.3	(14.1, 128.3)	55/80, 68.8%	27.3 ± 15.9	(13.8, 128.3)	178/240, 74.2%
naringenin	19.8 ± 52.2	(0.0, 357.0)	55/80, 68.8%	9.5 ± 17.8	(0.0, 123.6)	59/80, 73.8%	12.2 ± 21.0	(0.0, 127.5)	58/80, 72.5%	13.7 ± 33.7	(0.0, 357.0)	172/240, 71.7%
hesperetin	37.6 ± 74.0	(0.9, 437.6)	47/80, 58.8%	14.0 ± 22.3	(1.4, 136.4)	56/80, 70.0%	19.3 ± 61.9	(1.2, 446.5)	54/80, 67.5%	22.9 ± 56.5	(0.9, 446.5)	157/240, 65.4%
total aglycones	121.3 ± 128.2	(19.0, 1007.4)	80/80, 100.0%	87.0 ± 52.1	(13.6, 335.9)	80/80, 100.0%	102.1 ± 90.5	(15.5, 716.6)	80/80, 100.0%	103.5 ± 96.1	(13.6, 1007.4)	240/240, 100.0%

the lack of literature on the subject. Further research on the presence of flavonoids in human milk should strongly consider a greater sample size and the use of dietary questionnaires to obtain more relevant flavonoid intake data.

4.3.2 Flavonoid Metabolites

The qualitative profiles and average concentrations of individual flavonoid metabolites were estimated by analyzing pooled human milk samples [**Table 4.4**]. Due to the lack of authentic flavonoid metabolite standards, a rodent plasma samples containing several of these metabolites were used to compare chromatograms and mass spectra in order to confirm their identity **[Appendix A]**. Metabolites that were not in plasma the rodent were tentatively identified based on their relative elution time and mass spectra. To our knowledge, this is the first report of flavonoid metabolites in human milk. Two different epicatechin glucuronides and a methylated glucuronide metabolite were detected in milk. Previous *in vivo* and *in vitro* studies determined that epicatechin metabolites include multiple methylated, glucuronidated, methyl-glucuronidated, and sulfonated conjugates.[236-238] Flavonols, quercetin and kaempferol, were present in human milk as either glucuronides or methylated glucuronides. However, multiple metabolites including diglucuronides, sulfates, and glucoside sulfates of quercetin and kaempferol have been previously detected in human urine.[239] Flavanones, hesperetin and naringenin, were present in human milk as glucuronides. Previous studies indicated that heseperetin and narigenin are conjugated as glucuronides and sulfates in rodents and humans.[240-242]

Table 4.4 Flavonoid Metabolite Average Concentration of Two Pooled Samples From Each Country by Lactation Stage (nmol/L)

			China				**USA**				**Mexico**			
	ESI- fragment m/z		*week*				*Week*				*week*			
Compound	**M-**	**F-**	**2**	**4**	**13**	**26**	**2**	**4**	**13**	**26**	**2**	**4**	**13**	**26**
epicatechin glucuronide A	465	375, **289**, 255	3.93	3.52	3.13	2.03	2.65	1.81	1.99	2.33	1.46	1.62	1.49	1.07
epicatechin glucuronide B	465	375, **289**, 255	1.00	0.91	1.46	0.70	0.49	0.09	0.04	0.04	0.25	0.12	ND	0.04
epicetchin methyl-O-glucuronide	479	**289**, 193	5.18	7.44	3.42	6.19	0.72	1.02	0.83	ND	ND	0.70	0.72	0.96
naringenin glucuronide	447	367, 212	0.17	0.10	ND	0.19	0.55	0.31	ND	0.20	0.44	ND	ND	ND
kaempferol glucuronide*	461	429, 336, 315, 187	6.53	7.32	15.53	14.09	5.38	2.15	3.98	2.61	5.17	3.04	2.38	1.58
hesperetin glucuronide	477	390, **301**	3.57	4.48	3.98	2.84	0.36	ND	0.32	0.21	0.22	0.18	ND	0.14
quercetin glucuronide	477	356, **301**	3.84	4.06	4.53	4.04	1.73	1.93	1.82	2.11	1.50	1.70	2.15	1.99
quercetin Me-O-glucuronide*	491	427, 356, **301**	3.17	3.02	2.74	3.07	ND	ND	ND	ND	ND	ND	ND	ND

Interestingly, sulfonated metabolites of any flavonoid were not detected in any milk sample. This may indicate that sulfonated metabolites may not be transported into the mammary glands. However, it is also possible that the extraction methodology utilized in this study was not selective for these metabolites which can be notoriously difficult to extract under broad conditions used in such screening.[243] Further research concerning flavonoid metabolites in human milk may provide a deeper understanding of their presence and elimination.

4.3.3 Anthocyanin Content

A preliminary scan for anthocyanins and anthocyanin metabolites was conducted. However, no anthocyanins or their metabolites were detected in human milk samples. This result is likely related to the relatively low bioavailability of anthocyanins.[113-114] For instance, urinary excretion of delphinidin, and cyanidin glucodies and rutinosides were between 0.007 and 0.133% of the intake dose.[114] In addition, the maximum plasma concentration of malvidin 3-O-glucoside after red wine consumption reached 0.001 µM.[113] Furthermore, anthocyanins are generally not considered a major contributor to the composition of dietary flavonoids, despite their high color impact to foods. Multiple surveys suggested that anthocyanins only comprise of 1.6 to 6.0% of consumed flavonoids in The United States and in Spain. [16-18] Therefore, concentrations of anthocyanins below the analytical limit of detection, approximately 0.04 nmol/L milk, is reasonable to expect. For instance, rats exposed to anthocyanin rich diets for 15 days contained 0.05 and 0.21 nmol cyanidin 3-O-glucoside per gram of liver and brain, respectively.[120] Furthermore, after a single dose of pelargonidin was administered to rats,

0.5 and 0.15 nmol of pelargonidin glucuronide was detected per gram of kidney and liver, respectively.[244] Future studies assessing anthocyanin content of human milk form free living mother would therefore need higher sample volumes than the 2 mL used in these studies. High volumes does increase the complexity and cost of associated studies and would make broad screenings, such as those applied in this study, not feasible for most investigations.

4.4 Conclusion

Epicatechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate, naringenin, kaempferol, hesperetin, and quercetin were detected in human milk from cohorts in China, Mexico, and The Unites States. Milk from China had significantly greater total flavonoid aglycone content in comparison to the American cohort. The Mexican and Chinese cohorts had significantly higher concentrations of epicatechin gallate and than kaempferol than the American cohort, respectively. Furthermore, glurcuronide metabolites of the flavonoids were detected in milk samples. This research expands on the limit knowledge of the presence of flavonoids in human milk.

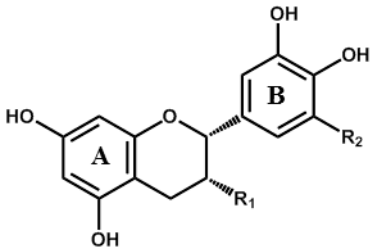
CHAPTER 5. THE DEGRADATION OF GREEN TEA FLAVAN-3-OLS AND THE FORMATION OF HETERO- AND HOMOCATECHIN DIMERS IN MODEL DAIRY BEVERAGES

5.1 Introduction

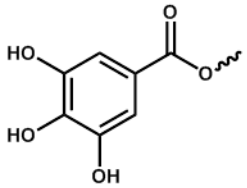
Teas are one of the most commonly consumed beverages worldwide. Green tea contains high concentrations of polyphenols, predominantly composed of flavan-3-ol monomers including epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG). Flavan-3-ols have a generic backbone while the presence of a galloyl moiety or an additional hydroxy substitution on the B-ring determines the exact compound **[Figure 5.1]**. Diets rich in flavan-3-ols have been associated with reduced risk of chronic diseases including cardiovascular diseases, certain cancers, diabetes and obesity.[195, 245-247] Therefore, the bioavailability, chemistry, and physiological responses of dietary flavan-3-ols have been the focus of recent studies. However, they are vulnerable to several degradative reactions accelerated by heat, food ingredients, elevated pH (>5) conditions, and presence of dissolved oxygen or other reactive oxygen species.[30, 128, 143] Although there are many mechanisms of degradation, the formation of simple auto-oxidation products including theasinesin (THSN) and P-2 dimers has been the focus of recent studies **[Figure 5.2]**.[248] In general, the auto-oxidation of flavan-3-ols occurs by the formation of a radical semiquinone structure and is stabilized via multiple resonance structures available to the flavan-3-ol.[129] Further

oxidation will result in the formation of a stabilized quinone structure.[130-131] Once flavan-3-ol quinones are formed, they may react with other flavan-3-ols or quinones to produce a THSN dimer or its quinone analogue, respectively.[133] Additional oxidation of the THSN dimer will produce its quinone analogue as well, which can then be further oxidized to form the P-2 dimer.[133] The dimers have been shown to be naturally present in oolong and black teas and also form during storage and digestion of green tea.[132] Furthermore, studies have investigated the bioavailability of the dimers in both rodent and cell culture models demonstrating their potential biological relevance.[29, 249] However, to date, preservation of monomeric forms is believed to be more critical to delivery of health benefits.

One factor believed to alter stability and bioavailability of flavan-3-ols is the presence of milk protein. Polyphenols have previously been reported to interact with macromolecules in the food matrix or in vivo. For instance, Bennick et al. described interactions between polyphenols and salivary proteins as a main contributor to an



Epicatechins

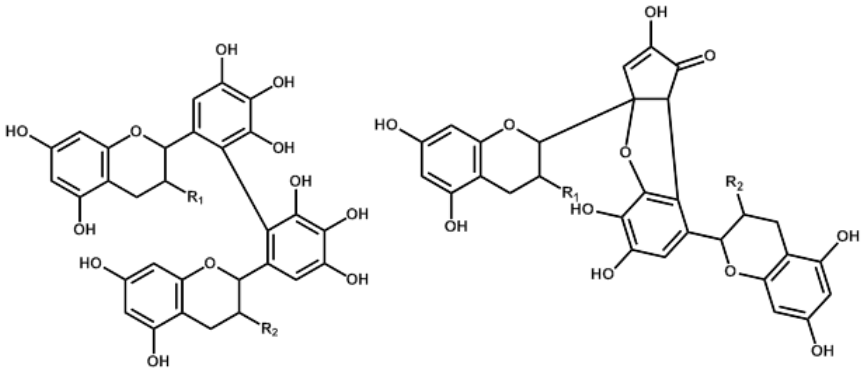


Galloyl Moiety

Compound	m/z	Abbreviation	R1	R2
Epicatechin	289	EC	OH	H
Epigallocatechin	305	EGC	OH	OH
Epicatechin gallate	441	ECG	Galloyl	H
Epigallocatechin gallate	457	EGCG	Galloyl	OH

Figure 5.1 Structure of Monomeric Flavan-3-ols.

astringent sensation.[169] A molecular model for astringency based on protein-polyphenol interactions has been described.[170] These interactions are not isolated to the consumption of polyphenol rich beverages as they can also occur during storage and production. Siebert et al. investigated protein-polyphenol interaction and the relation to haze formation in beer, wine, and apple juice.[250] Hydrophobic and hydrogen bonding forces between polyphenols and proline rich segments have been reported to be important driving factors for these interactions.[176] Thus, proline rich proteins including β-casein and salivary proteins have been targeted by recent studies. However, studies have not investigated the impact of these interactions on the stability of flavan-3-ols and their potential involvement in modulation of auto-oxidation reactions in food systems.



THSNs **P-2 and P-2 analogs**

Compound	Monomer Composition	m/z	R1	R2
THSN A/D	2 EGCG	913	Galloyl	Galloyl
THSN C/E	2EGC	609	OH	OH
THSN B	EGCG + EGC	761	Galloyl	OH
P-2	2 EGCG	883	Galloyl	Galloyl
P-2 analog	2 EGC	579	OH	OH
P-2 analog	EGCG + EGC	731	Galloyl/OH	OH/Galloyl

Figure 5.2 Structure of THSN and P-2 Dimers.

Recently, our research group investigated the presence of monomeric flavan-3-ols in human milk from 17 mothers during 3 lactation stages.[228] EC, ECG, and EGCG were detected in milk samples at 63.7 - 828.5, 55.7 - 645.6, and 215.1 - 2364.7 nmol/L, respectively. In contrast, EGC was not detected in any of the milk samples and its absence is caused, in part, by dietary habits as well as the chemistry of EGC. For instance, EGC is more susceptible to degradative reactions than EC, ECG, and EGCG.[143] Furthermore, EGC has a lower binding affinity to milk and human serum proteins than ECG and EGCG.[173, 251] The potential for flavan-3-ol binding may limit their availability to oxidative reactions and thus alter the stability of monomeric flavan-3-ol. Therefore, ECG may be more likely to degrade below the analytical limit of detection before ECG or EGCG in a protein rich matrix. However, the degradation of monomeric flavan-3-ols in milk protein rich model beverages has not been fully investigated. The current study investigates the degradation of monomeric flavan-3-ols and the formation of dimers at multiple polyphenol to protein ratios in both a model dairy system at human body (37^{o}C) and batch pasteurization (62^{o}C) temperatures.

5.2 Materials and Methods

5.2.1 Materials

L-ascorbic acid, Na_2-ethylenediaminetetraacetic acid (EDTA), pepsin (# P7000), citric acid, sodium phosphate dibasic, formic acid, EC, EGC, ECG, and EGCG were purchased from Sigma Aldrich (St. Louis, MO). Solvents including mass spectroscopy grade water, methanol, isopropanol, and HCl were purchased from Mallinckrodt Baker (Phillipsburg, NJ). Green tea extract (GTE) and Teavigo®, >94% EGCG concentrate,

were donated to our research group from Nestlé and DSM, respectively. Saco® (Middleton, WI) non-fat dry milk (NFDM) was purchased from a local market.

5.2.2 Model Dairy Beverages

A range of polyphenols and model dairy beverages were selected in order to investigate multiple polyphenol to protein ratios. 10% and 2.5% w/w NFDM in double distilled water were used as our model dairy beverages corresponding to single strength milk (SSM) and quarter strength milk (QSM), respectively. These preparations delivered approximately 36.2 and 9.0 g protein per L finished beverage, respectively. NFDM concentrations were selected to approximate the protein content of dairy beverages.[252] A 0% w/w NFDM buffered to pH 6.30 ± 0.02 using citric acid and sodium phosphate dibasic was used as a zero protein control (ZPC). GTE were dosed into model dairy beverages at 500, 100, 0.5, and 0.1 mg/L and Teavigo® was dosed into model dairy beverages at 100, 10, 1, and 0.1 μM. EC, EGC, ECG, and EGCG were present in the GTE at 37.4, 40.4, 196.9, and 240.1 μmol/g. Thus, 500 mg/L of GTE was equivalent to 18.7, 20.2, 48.5, and 120.1 μM of EC, EGC, ECG, and EGCG, respectively. Similarly, 100, 0.5 and 0.1 mg/L GTE solution contained the same ratio of flavan-3-ols at their equivalent dilutions. No detectable quantities of THSN or P-2 dimers were present in stock GTE when immediately stabilized in 2% acetic acid in water. These results confirm that the GTE extract used in this study is a lightly processed an minimally oxidized green tea. The wide range in of GTE and Teavigo concentrations were used to approximate EGCG concentrations found in both teas and ready to drink tea beverages (2.62 - 283 μM) and human milk (0.215 - 2.36 μM).[30, 228]

5.2.3 Kinetics

Aliquots of model beverages were incubated at 37 and 62^{o}C in a water bath in order to calculate kinetic degradation parameters relevant to shelf stability, respectively. Samples were retrieved at 0, 30, 60, 90, 120, 150, and 180 minutes. Flavan-3-ols have been reported to follow first order degradation kinetics under thermal stress.[157] Thus, degradation rates were calculated by utilizing a linear regression model of natural $\log(C_x/C_o)$ vs. time (minutes), where C_x and C_o are the concentration of a flavan-3-ol at a given time and initial time, respectively. The negative slope of the linear model was then recorded as the first order degradation rate constant.

5.2.4 Extraction and Analysis

All samples were extracted as described by Song et al. with minor modifications [228]. Briefly, samples were removed from the water bath and immediately chilled on ice. 50 µl of 2.7 mM L-ascorbic acid and 2.2 mM Na_2-EDTA in water were added followed by 6 mL of 40 mg/mL pepsin in 0.1 N HCl. Samples were incubated for 15 minutes at 37^{o}C with mild agitation. No β-glucuronidase with sulfatase contaminant was used in this extraction as these were unmetabolized non-physiological samples. Flavan-3-ols were then extracted with ethyl acetate and evaporated under nitrogen. The remaining solid extract was dissolved in 0.4% formic acid in water, centrifuged, and transferred into LC vials.

An Agilent (Santa Clara, CA) series 1100 HPLC equipped with a Xterra Reversed Phase C18 column (3.5 um 2.1 x 100 mm) (Milford, MA) was used for all chromatographic separations. A binary gradient consisting of mobile phase A (0.4%

formic acid in water) and B (4% isopropanol and 0.4% formic acid in methanol) was used. Initial conditions were set to 15% B and transitioned to 100% B at 9.5 minutes where it remained isocratic for 1 minute before rapidly resetting to 15% B at 20 minutes. Following separation, column effluent was passed to a Waters (Milford, MA) LCT Premier time of flight mass spectrometer. The electrospray ionization was set to negative mode and programmed to scan from 100–1000 m/z in V-mode. Capillary and cone voltages were set to 3500 and 35 volts, respectively. Desolvation and source temperatures were set to 250 and 150^{o}C, respectively. Cone and desolvation nitrogen gas flow rates were set to 60 and 400L/hour. EC, EGC, ECG, and EGCG were detected at 289, 305, 441, and 457 m/z, respectively **[Figure 5.1]**. Dimers were detected at 579, 609, 731, 761, 883, and 913 m/z **[Figure 5.2]**.

5.2.5 Statistical Methods

All combinations of temperatures, NFDM concentrations, and flavan-3-ol concentrations were run in triplicates. First order degradation rates were calculated by utilizing a linear regression model of natural log(C_x/C_o) vs. time (minutes), to calculate the slope (min^{-1}). Significant differences between degradation rates and concentrations were identified by Tukey's post hoc tests (α=0.05). All degradation rates are expressed as mean ± standard deviation (n=3).

5.3 Results and Discussion

5.3.1 Isolated EGCG Degradation Kinetics in Model Dairy Beverages

In general, decreasing protein concentration significantly increased the degradation rates of EGCG at both 37°C and 62°C. For instance, the degradation rate of 100μM EGCG at 62°C significantly ($P<0.05$) increased from $2.83x10^{-3}$ to $4.15 x10^{-3}$ to $6.67 x10^{-3}$ min^{-1} between SSM, QSM, and ZPC, respectively **[Table 5.1]**. Similar significant differences in reaction rates were observed for 10, 1, and 0.1μM EGCG concentrations at 62°C. At 37°C, EGCG at any concentration had significantly lower degradation rates in the SSM than both QSM and ZPC model beverages. However, at 100 and 10 μM EGCG, there was no significant ($P>0.05$) difference between QSM and ZPC **[Table 5.2]**. The majority of proteins in the bovine milk are α- and β-casein, both of which contain relatively high proline content [253]. Previous studies have demonstrated the ability of polyphenols including green tea flavan-3-ols to bind to proline rich proteins including caseins.[165, 174-175] More recently, Bohin et al. demonstrated that amino acid composition and protein structure determine the availability of proline and aromatic amino acids, which impacts these interactions.[168] Furthermore, the elevated temperatures and drying conditions associated with NFDM production alters casein micelle structures

Table 5.1 First Order Degradation Rates (min^{-1}) of EGCG at 62°C.

	100μM EGCG	**10μM EGCG**	**1μM EGCG**	**0.1μM EGCG**
SSM	2.83E-3 ± 3.0E-4^{A1}	2.26E-3 ± 5.1E-4^{A1}	1.31E-3 ± 6.9E-4^{B1}	1.32E-3 ± 1.5E-4^{B1}
QSM	4.15E-3 ± 5.2E-4^{A2}	4.50E-3 ± 5.0E-4^{A2}	3.26E-3 ± 4.1E-4^{B2}	3.24E-3 ± 1.1E-4^{B2}
ZPC	8.67E-3 ± 6.6E-4^{A3}	8.45E-3 ± 4.6E-4^{A3}	8.96E-3 ± 8.9E-4^{AB3}	9.62E-3 ± 6.2E-4^{B3}

Data is expressed as mean ± standard deviation. Single strength milk (10% non-fat dry milk), quarter strength milk (2.5% non-fat dry milk), and zero protein control (0% non-fat dry milk) are abbreviated as SSM, QSM, and ZPC, respectively. Different superscript letters indicated significant differences between EGCG concentrations with a model dairy beverage. Different superscript numbers indicate significant differences between model dairy beverages within EGCG concentrations.

when reconstituted.[254] Thus, the structure of casein micelles and the availability of β-casein will influence the effective binding with EGCG. Nevertheless, polyphenols bound to proteins or within protein capsules have been shown to have greater stability. Shpigelman et al. demonstrated that thermally treated β-lactoglobulin can form nanocomplexes to protect EGCG from degradation.[167] Although a mixture of milk proteins were used in our study, the concept that polyphenols may be stabilized by interacting with proteins is well illustrated. Therefore, the significant decrease in EGCG degradation rates with increasing protein concentration in model dairy beverages is likely related to the binding of polyphenols to proline rich proteins.

Previous experiments have suggested that non-covalent interactions including hydrophobic interactions and hydrogen bonding forces are the driving forces between polyphenols and proteins.[165] However, more recent studies strongly suggest that hydrophobic interactions are more predominant than hydrogen bonding forces.[175, 177, 255] At elevated temperatures, both non-covalent interactions are weakened. As a result, bound EGCG molecules are more likely to be released from the protein and into solution. Therefore, assuming the presence of significant protein-EGCG interactions, the increase in degradation rates at greater temperature is caused not only by typical Arrhenius behavior of EGCG, but likely also the weakening of non-covalent interactions between milk proteins and EGCG. Under these conditions, the frequency factor in the Arrhenius model is not a constant and is likely to increase with greater temperatures. Therefore, EGCG degradation in milk protein solutions will not accurately fit the Arrhenius model.

Within the SSM and QSM model beverages, EGCG degradation rates significantly decreased with decreasing EGCG concentrations at 37 and 62^{o}C. For

example, the degradation rates significantly decreased at 37°C from $5.83x10^{-4}$ to $5.31x10^{-4}$ min^{-1} in SSM models between 100μM and 100nM EGCG concentrations, respectively **[Table 5.2]**. Bohin et al. calculated that a maximum of 38.1 g and 16.5 g of EGCG may bind with 100g of β-casein and β-lactoglobulin, respectively.[168] SSM and QSM contained 36.2 and 9.0 g protein per L finished beverage and milk proteins are approximately composed of ~28 and 12% β-casein and β-lactoglobulin, respectively.[256] Thus, SSM approximately contains 10.14g β-casein and 4.34g β-lactoglobulin, which may bind 3.86g and 0.72g EGCG per liter. Therefore, SSM and QSM model beverages would be saturated at approximately 1.00 and 0.25 mM EGCG, respectively. The highest concentration of EGCG used in present experiments was 0.1 mM, far lower than the theoretical saturation points for either protein containing model beverage. Therefore, it is likely that the proteins in our model milk beverages did not approach saturation with EGCG, suggesting physical stability was not a factor in the observed losses.

A protein-polyphenol interaction model described by Siebert et al. rationalized that the ratio of polyphenols to their potential binding sites on proteins significantly impact these interactions and may be characterized by three scenarios.[165] If the quantity of polyphenol binding sites on proteins is roughly equivalent to the concentration of

Table 5.2 First Order Degradation Rates (min^{-1}) of EGCG at 37°C.

	100μM EGCG	10μM EGCG	1μM EGCG	0.1μM EGCG
SSM	5.83E-4 ± 3.0E-5^{A1}	5.66E-4 ± 2.8E-5^{AB1}	5.34E-4 ± 3.4E-5^{B1}	5.31E-4 ± 3.2E-5^{B1}
QSM	4.05E-3 ± 4.2E-4^{A2}	3.83E-3 ± 6.8E-4^{AB2}	3.25E-3 ± 3.5E-4^{B2}	3.32E-3 ± 4.5E-4^{B2}
ZPC	4.58E-3 ± 3.6E-4^{A2}	4.62E-3 ± 6.5E-4^{AB2}	4.86E-3 ± 1.0E-3^{AB3}	5.29E-3 ± 3.4E-4^{B3}

Data is expressed as mean ± standard deviation. Single strength milk (10% non-fat dry milk), quarter strength milk (2.5% non-fat dry milk), and zero protein control (0% non-fat dry milk) are abbreviated as SSM, QSM, and ZPC, respectively. Different superscript letters indicated significant differences between EGCG concentrations with a model dairy beverage. Different superscript numbers indicate significant differences between model dairy beverages within EGCG concentrations.

polyphenols, large complexes may form, resulting in precipitation and loss of stability. An excess of either proteins or polyphenols will decrease the size of the complex and avoid precipitation. In our experiments, the proteins were never saturated with polyphenols, thus the loss of EGCG stability was predominately caused by oxidation and not by precipitation. This was confirmed by a lack of precipitate in the model beverage systems both before and after treatment. Furthermore, EGCG was titrated into SSM and no precipitants were observed from 0 to 500μM EGCG. Decreasing the polyphenol to protein ratio will increase the probability that a free EGCG molecule will interact with an available binding site on a protein. Thus, EGCG degradation rates significantly decreased with decreasing concentrations of EGCG within a given protein containing model beverage. Similarly, decreasing protein concentrations within a constant EGCG concentration significant increased EGCG degradation rates likely because of the decrease in number polyphenol binding sites on available proteins.

The relative concentrations of THSN A/D and P-2 dimers were monitored at each time point. Presence of these oxidation products provide insight in to auto-oxidative mechanisms of EGCG degradation. However, their kinetics could not be accurately modeled because of the simultaneous formation and multiple degradation reactions of each dimer. Detectable quantities of the dimers formed at most time points in QSM and ZPC models containing 100uM EGCG **[Figure 5.3]**. No dimer formation at any time point was observed in any SSM model. This is likely caused, in part, by the increased concentration of proteins enabling more EGCG binding and preventing their formation of subsequent oxidation products including dimers. At lower protein concentrations, polyphenol binding sites are more limited, thus EGCG is likely distributed in both bound

and unbound forms and may proceed with the auto-oxidation mechanism leading to more complex product formation including dimers. Although the enhanced stability of EGCG at higher protein concentrations suggests that proteins directly inhibit EGCG oxidation, the inhibition of THSN A/D formation may involve other mechanisms. For instance, the formation of THSN A/D requires the initial oxidation of EGCG to its quinone analogue.[129-131, 133] The EGCG quinone is structurally similar to its precursor because the A-ring, C-ring, and the galloyl moiety are not disrupted. Therefore, the quinone may be able to bind to proteins through hydrophobic and hydrogen bonding interactions. However, the B-ring on EGCG quinone is rendered non-aromatic and can no longer function as hydrogen bond donors. Therefore, their binding to proline rich segments of proteins is likely to be weaker relative to EGCG. Furthermore, quinones are able to react directly to proteins, which may contribute to non-dimer related degradation. For instance,

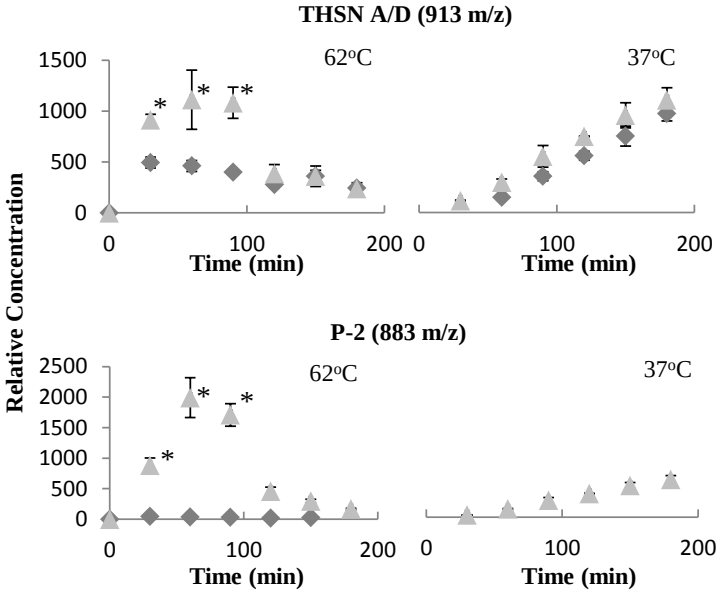


Figure 5.3 THSN A/D and EGCG P-2 homodimer formation in 100uM isolated EGCG solutions.

♦ and ▲ designate QSM and ZPC, respectively. No dimers were detected in the SSM model. * indicates significant differences in dimer concentration within a given time point between model dairy beverages.

p-quinones may covalently bond to cysteine residues by a thiol addition mechanism.[257-259] However, our analysis was not optimized to detect these compounds, thus more research may be needed to elaborate on this mechanism.

In contrast to THSN A/D, the P-2 dimer was not detected in QSM model at 37°C but was detected at low concentrations at 62°C. Considering that precursors for both dimerization reactions are the same, THSN A/D was readily formed under these conditions, and the formation of both dimers in the ZPC at both temperatures, it is likely that the mechanism for the P-2 dimer formation was specifically inhibited by the protein. As previously discussed, intermediate compounds in the auto-oxidation process, may be bound to proteins and thus inhibit the formation of further oxidation products. Furthermore, the binding constants of procyanidins, polymeric flavan-3-ols, to proteins increased with degree of polymerization.[260] This may indicate that either THSN A/D or its quinone analogue are bound to proteins after their formation.[133] Previous research suggests that THSN serves as a precursor for the formation of the P-2 dimer.[261] Thus, the significantly lower P-2 dimer formation with increased protein concentration is in part caused by the binding of THSN.

5.3.2 Impact of Flavan-3-ol Structure on Interactions with Proteins

The presence of the hydroxyl and galloyl moieties had varying impacts on the degradation rates of flavan-3-ols from GTE in model dairy beverages. The degradation rate of EGC was significantly lower in the SSM in comparison to either QSM and ZPC at 62 and 37°C in 20.2 and 4.0 μM EGC solutions **[Table 5.3 and 5.4]**. Similarly, ECG degradation rates significantly decreased with increasing protein concentration. However,

Table 5.3 First Order Degradation Rates (min^{-1}) of Flavan-3-ols in GTE at 62°C.

Epicatechin

	18.7 µM	3.7 µM	18.7 nM	3.7 nM
SSM	1.19E-3 ± 4.7E-4^{A1}	1.12E-3 ± 6.7E-4^{A1}	1.38E-3 ± 3.1E-4^{A1}	1.27E-3 ± 4.7E-4^{A1}
QSM	1.15E-3 ± 3.1E-4^{A1}	1.16E-3 ± 3.1E-4^{A1}	1.00E-3 ± 3.3E-4^{A1}	1.35E-3 ± 3.4E-4^{A1}
ZPC	1.25E-3 ± 3.9E-4^{A1}	1.13E-3 ± 1.9E-4^{A1}	1.19E-3 ± 3.1E-4^{A1}	1.31E-3 ± 8.1E-4^{A1}

Epigallocatechin

	20.2 µM	4.0 µM	20.2 nM	4.0 nM
SSM	3.47E-3 ± 3.5E-4^{A1}	3.31E-3 ± 5.4E-4^{A1}	4.47E-2 ± 3.7E-3^{B1}	4.84E-2 ± 4.1E-3^{B1}
QSM	1.40E-2 ± 5.9E-3^{A2}	1.24E-2 ± 2.6E-3^{A2}	4.31E-2 ± 1.4E-3^{B1}	4.44E-2 ± 5.8E-3^{B1}
ZPC	2.85E-2 ± 6.1E-3^{A3}	3.06E-2 ± 1.3E-3^{A3}	4.41E-2 ± 4.2E-3^{B1}	4.35E-2 ± 3.6E-3^{B1}

Epicatechin Gallate

	48.5 µM	9.7 µM	48.5 nM	9.7 nM
SSM	9.73E-4 ± 3.8E-5^{A1}	8.56E-4 ± 5.4E-5^{B1}	8.54E-4 ± 6.4E-5^{B1}	8.77E-4 ± 5.8E-5^{B1}
QSM	1.21E-3 ± 2.6E-4^{A12}	1.24E-3 ± 4.4E-4^{A12}	9.85E-4 ± 1.1E-4^{AB1}	9.24E-4 ± 6.2E-5^{B1}
ZPC	1.62E-3 ± 3.8E-4^{A2}	1.89E-3 ± 4.7E-4^{A2}	1.77E-3 ± 3.4E-4^{A2}	1.74E-3 ± 1.3E-4^{A2}

Epigallocatechin Gallate

	120.1 µM	24.0 µM	120.1 nM	24.0 nM
SSM	2.91E-3 ± 2.8E-4^{A1}	2.61E-3 ± 5.7E-4^{A1}	1.92E-3 ± 2.5E-4^{B1}	1.95E-3 ± 1.0E-4^{B1}
QSM	6.59E-3 ± 7.9E-4^{A2}	6.24E-3 ± 6.9E-4^{A2}	4.11E-3 ± 5.7E-4^{B2}	4.45E-3 ± 6.9E-4^{B2}
ZPC	2.60E-2 ± 3.2E-3^{A3}	2.91E-2 ± 1.4E-3^{A3}	4.04E-2 ± 3.4E-3^{B3}	3.88E-2 ± 1.2E-3^{B3}

Data is expressed as mean ± standard deviation. Single strength milk (10% non-fat dry milk), quarter strength milk (2.5% non-fat dry milk), and zero protein control (0% non-fat dry milk) are abbreviated as SSM, QSM, and ZPC, respectively. Different superscript letters indicated significant differences between flavan-3-ol concentrations with a model dairy beverage. Different superscript letters indicated significant differences between flavan-3-ol concentrations with a model dairy beverage. Different superscript numbers indicate significant differences between model dairy beverages within a flavan-3-ol concentration.

EC degradation rates showed no significant differences between any model dairy beverages. Interestingly, in μM range concentrations, significant differences in degradation rates between SSM and ZPC were observed for ECG, but not EGC, at 62 and 37^{o}C. This indicates that the presence of the galloyl moiety at the C3 position influences polyphenol to protein interactions to a greater extent than the presence of the hydroxyl moiety on the B-ring. Thus, the C3 galloyl moiety greatly enhances the binding of flavan-3-ols to proteins and protects them from degradation. Our results are in agreement with previous studies that differentiated the binding of the various flavan-3-ols and proteins. Minoda et al. and Xiao et al. demonstrated that C3 gallated flavan-3-ols bind more strongly than non-gallated flavan-3-ols to human serum albumin and milk protein, respectively.[173, 251] Furthermore, Hasni et al. determined that the EGCG has a greater binding constant and forms a more stable complex with α- and β-casein than EGC.[176] In addition, Hasni et al. determined that EGCG has a greater number of bound polyphenol per β-casein than C, EC, and EGC. These observations are likely attributed to the presence of the galloyl moiety on EGCG and its interactions with proline on β-casein.

Significant differences for EGCG from GTE degradation rates across model dairy beverages were similar to those observed in kinetics studies involving isolated EGCG described previously. For example, degradation rate at 62^{o}C in 24.0 μM EGCG solutions significantly increase from 2.61×10^{-3} to 2.91×10^{-2} min^{-1} in SSM and ZPC, respectively **[Table 5.3]**. Furthermore, at 37^{o}C in 120.1 nM EGCG solutions degradation rates significantly increase from 4.07×10^{-3} to 1.24×10^{-2} min^{-1} between SSM and ZPC models **[Table 5.4]**. As previously discussed, ECG and EGC are also capable of interacting with

Table 5.4 First Order Degradation Rates (min^{-1}) of Flavan-3-ols in GTE at 37°C

	Epicatechin			
	18.7 µM	**3.7 µM**	**18.7 nM**	**3.7 nM**
SSM	5.68E-4 ± 4.7E-5^{A1}	5.49E-4 ± 5.9E-5^{A1}	5.92E-4 ± 6.0E-5^{A1}	5.75E-4 ± 3.3E-5^{A1}
QSM	5.70E-4 ± 4.9E-5^{A1}	5.34E-4 ± 6.9E-5^{A1}	5.71E-4 ± 4.5E-5^{A1}	5.54E-4 ± 4.6E-5^{A1}
ZPC	5.45E-4 ± 3.2E-5^{A1}	5.52E-4 ± 2.3E-5^{A1}	5.83E-4 ± 3.7E-5^{A1}	5.61E-4 ± 3.8E-5^{A1}
	Epigallocatechin			
	20.2 µM	**4.0 µM**	**20.2 nM**	**4.0 nM**
SSM	6.82E-3 ± 2.6E-4^{A1}	6.53E-3 ± 4.0E-4^{A1}	9.56E-3 ± 3.5E-4^{B1}	9.34E-3 ± 6.6E-4^{B1}
QSM	8.01E-3 ± 2.3E-4^{A2}	7.98E-3 ± 5.6E-4^{A2}	9.41E-3 ± 3.5E-4^{B1}	8.99E-3 ± 4.1E-4^{B1}
ZPC	8.31E-3 ± 2.0E-4^{A2}	8.38E-3 ± 4.4E-4^{A2}	9.54E-3 ± 4.1E-4^{B1}	9.30E-3 ± 3.5E-4^{B1}
	Epicatechin Gallate			
	48.5 µM	**9.7 µM**	**48.5 nM**	**9.7 nM**
SSM	4.67E-4 ± 3.7E-5^{A1}	4.47E-4 ± 4.1E-5^{A1}	3.39E-4 ± 5.1E-5^{B1}	3.32E-4 ± 2.6E-5^{B1}
QSM	4.87E-4 ± 6.3E-5^{A12}	4.90E-4 ± 5.2E-5^{A12}	3.46E-4 ± 3.5E-5^{B1}	3.51E-4 ± 6.3E-5^{B1}
ZPC	5.20E-4 ± 2.2E-5^{A2}	5.53E-4 ± 3.7E-5^{A2}	7.75E-4 ± 6.3E-5^{B2}	7.43E-4 ± 5.1E-5^{B2}
	Epigallocatechin Gallate			
	120.1 µM	**24.0 µM**	**120.1 nM**	**24.0 nM**
SSM	6.88E-3 ± 3.9E-4^{A1}	6.43E-3 ± 3.2E-4^{A1}	4.07E-3 ± 6.2E-4^{B1}	4.11E-3 ± 5.5E-4^{B1}
QSM	7.93E-3 ± 5.9E-4^{A2}	7.87E-3 ± 5.5E-4^{A2}	6.16E-3 ± 1.3E-4^{B2}	6.38E-3 ± 3.7E-4^{B2}
ZPC	8.28E-3 ± 6.7E-4^{A2}	8.25E-3 ± 4.3E-4^{A2}	1.24E-2 ± 2.7E-3^{B3}	1.19E-2 ± 1.2E-3^{B3}

Data is expressed as mean ± standard deviation. Single strength milk (10% non-fat dry milk), quarter strength milk (2.5% non-fat dry milk), and zero protein control (0% non-fat dry milk) are abbreviated as SSM, QSM, and ZPC, respectively. Different superscript letters indicated significant differences between flavan-3-ol concentrations with a model dairy beverage. Different superscript letters indicated significant differences between flavan-3-ol concentrations with a model dairy beverage. Different superscript numbers indicate significant differences between model dairy beverages within a flavan-3-ol concentration.

proteins to produce a stabilizing effect. Considering that the GTE in the present study contained relatively high concentrations of EGCG, ECG, and EGC, the various flavan-3-ols may have competed with one another for binding sites on proteins. Thus, EGCG in a mixture of flavan-3-ols had a more limited availability of accessible binding sites due to competition with other flavan-3-ols thereby driving a larger portion to be unbound. This competition may have partially contributed to the lack of significant difference in EGC and ECG degradation rates in lower GTE concentration in model dairy beverages.

5.3.3 Formation of Flavan-3-ol Dimers in Model GT Milk Beverages

The relative concentration of various flavan-3-ol auto-oxidation dimers were monitored at each time point **[Figure 5.2]**. Similar to previous studies, EC or ECG homo- or heterodimers were not detected.[132] However, THSN A/D and EGCG P-2 homodimers in the GTE behaved similarly to the isolated EGCG systems between model beverages. For instance, in the 120.1 μM EGCG solution at 37°C, the P-2 dimer only formed in the ZPC, while the THSN A/D dimer was detected at greater quantities in the ZPC than the QSM model **[Figure 5.4]**. Furthermore, THSN A/D was detected in the SSM model at concentrations significantly ($P<0.05$) less than the two other model dairy beverages. These observations may be due, in part, by the presence of other flavan-3-ols, the reactivity of quinones to proteins, and the binding of THSN A/D proteins. As previously discussed, this may result in competition for binding sites, which can result in more unbound EGCG. Additionally, the presence of other flavan-3-ols allows for the potential of more diverse degradative pathways, like the formation of EGCG containing heterodimers which were not assayed in this study.

EGC formed a P-2 analogue and 3 different THSN C/E analogues corresponding to 579 and 609 m/z respectively. Neilson et al. rationalized that GTE would likely produce three different THSN C/E dimers because of the presence of EGC and the formation of GC via epimerization.[132] Thus, each of the detected peaks corresponds to THSN analogues of GC-GC, EGC-GC, and EGC-EGC. The concentrations of all three THSN C/E isomers did not significantly change between model dairy beverages at 62°C (data not shown). However, significant differences in THSN C/E isomers were found between model dairy beverages at 32°C **[Figure 5.5]**. The earliest eluting THSN C/E isomer (1.89 minutes) was not detected in the SSM model and significant differences occurred only at 60 and 180 minutes treatment time between QSM and ZPC. In contrast, significant differences in THSN C/E (retention times 2.53 and 3.34 minutes)

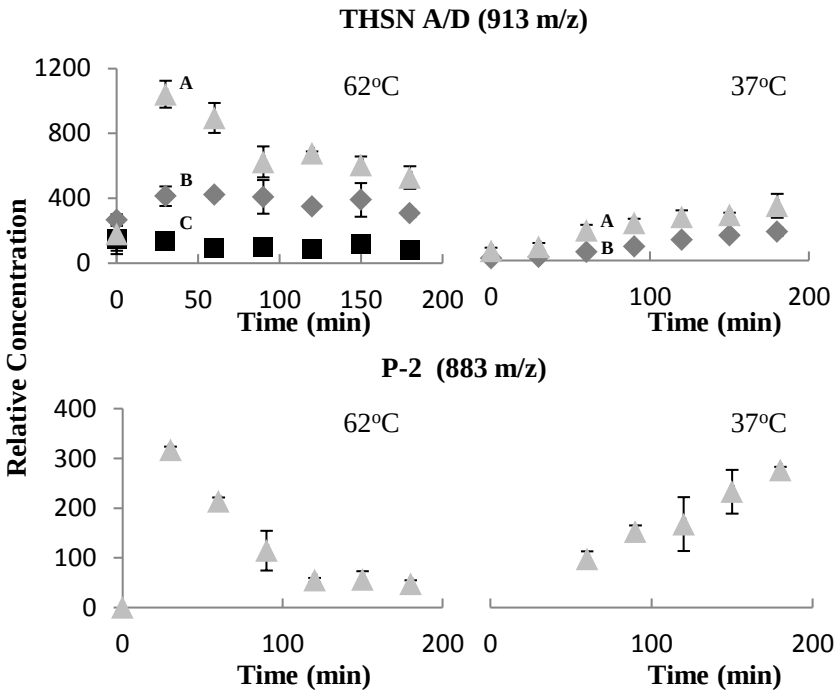


Figure 5.4 THSN A/D and EGCG P-2 Homodimer in 120.1 μM EGCG Solutions. ■, ♦, and ▲ designate SSM, QSM, and ZPC, respectively. Different letters indicate significant differences in dimer concentration between dairy models at all time points beyond and including the marked symbol.

concentrations occurred between the ZPC and all other model dairy beverages throughout the majority of the treatment time. Interestingly, THSN C/E dimers were detected at zero minutes and remain relatively constant throughout the incubation. Thus, it is likely that these dimers are formed instantly and are relatively stable at 37^{o}C. As previously described, EGC is known to bind with proteins, but the interaction is weaker in comparison to EGCG.[173, 251] Gallated flavan-3-ols preferentially bound to the proteins, limiting the available binding sites for EGC. Therefore, EGC is likely present more in unbound form and therefore available for reaction to readily form dimers regardless of protein concentration. However, because dimer formed in significantly higher concentration in the ZPC, some EGC may have been bound in both the QSM and SSM models.

THSN B and P-2 analogue heterodimers of EGCG and EGC were monitored at 761 and 731 m/z, respectively. THSN B formed in greater concentrations in the ZPC than the SSM at 37^{o}C and 62^{o}C **[Figure 5.6]**. However, there were generally no statistical differences between the ZPC and the QSM, with the exception of a few time points at 62^{o}C. Furthermore, the P-2 analogue of the EGCG-EGC heterodimer was only significantly different between the ZPC and all other model dairy beverages at 62^{o}C. Similar to the formation of THSN A/D and the P-2 homodimer, the majority of the EGCG was bound and unable to react in the SSM model. In addition, EGCG and EGC quinone intermediates may covalently bond to proteins in a non-dimer related degradation reaction. [257-259] Thus, one of the precursors for THSN B and the P-2 analogue was unavailable and limited the formation of the dimers. Additionally, the formation of the P-2 heterodimer only formed at 62^{o}C in the absence of protein.

Furthermore, polymeric flavan-3-ols have stronger binding affinities to proteins than their monomeric counterparts, thus THSN B was likely bound and unable to oxidize to the P-2 analogue. [260]

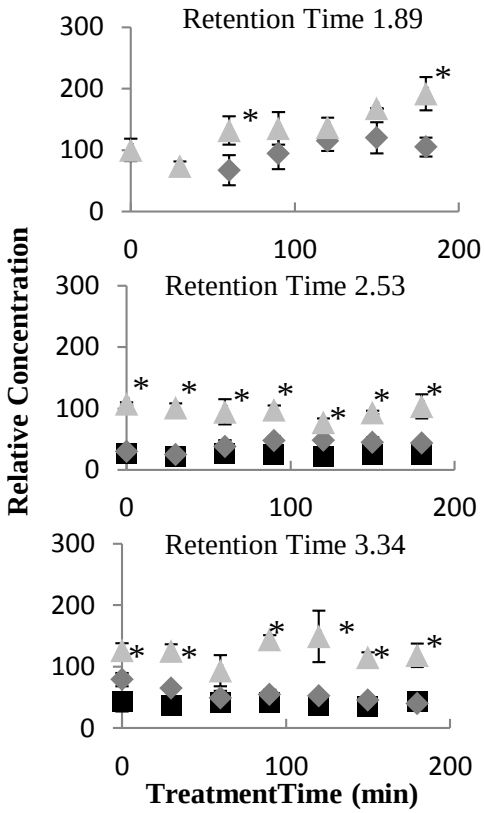


Figure 5.5 THSN C/E Isomers Formation at 1.89, 2.53, and 3.34 Minutes Retention Time in 20.2 μM EGC Solutions.

■, ♦, and ▲ designate SSM, QSM, and ZPC, respectively. * indicate significant differences between ZPC and all other plotted dimer concentrations at a given time.

5.4 Conclusion

In conclusion, greater polyphenol to protein ratios increased first order degradation rates, which also decreased the formation of THSNs and P-2 dimers. The mechanism includes the non-covalent binding of flavan-3-ols and auto-oxidation dimers to proteins and covalent bonding of quinones to proteins. The presence of the galloyl and hydroxyl moieties increased the binding of flavan-3-ols to proteins, thus stabilizing them with increasing protein concentrations. In contrast, the absence of these moieties led to no observable interactions to proteins.

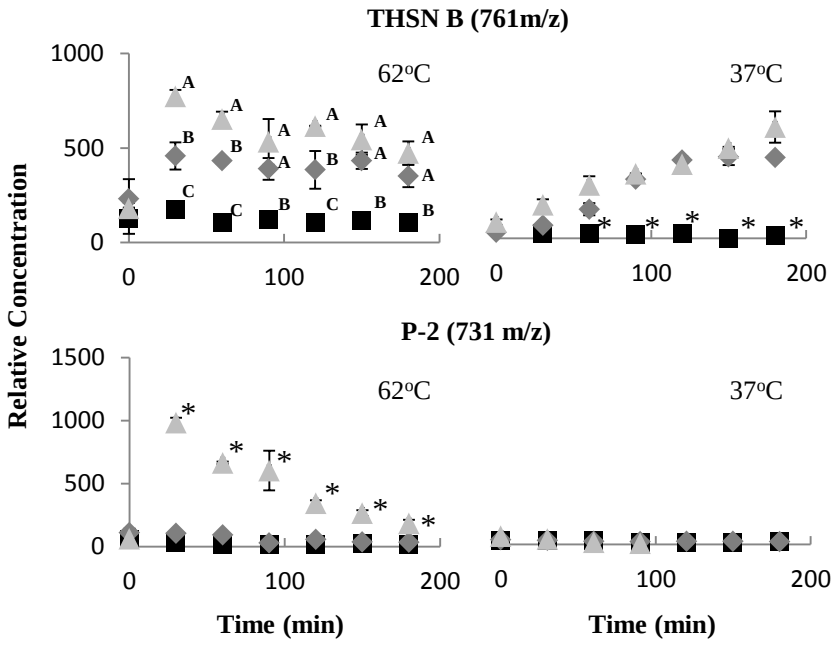


Figure 5.6 THSN B and EGCG-EGC P-2 Heterodimer Formation in 120.1 and 20.2 120.1 μM EGCG and EGC Solutions.

■, ♦, and ▲ designate SSM, QSM, and ZPC, respectively. Different letters indicate significant differences in dimer concentration within a given time point between dairy models. * indicate significant differences between marked model beverage and all other model beverages at a given timepoint.

CHAPTER 6. CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Introduction

The studies presented in this dissertation focused on flavonoid chemistry in model beverages, interaction with dairy proteins, and their presence in human milk samples longitudinally collected form free living mothers. Anthocyanin and flavan-3-ol kinetic degradation studies in model beverages highlighted the interactions between flavonoids, photo and thermal stress, and components in the food matrix. These interactions are typical of what may be encountered through food processing and storage thereby providing insight into factors driving stability and recovery of flavonoids in beverages systems encountered by consumers. The studies that focused flavonoid deposition in human milk significantly contributed to the knowledge of flavonoid tissue distribution and potential delivery to nursing infants. Furthermore, these studies provide a baseline for future development and assessment of polyphenols in infant formulas and foods. Although many questions were answered, the results of these studies justify further research concerning flavonoid chemistry in beverages and bioavailability. In the present chapter, potential future experiments and their significance are discussed

.

6.2 Anthocyanin Stability

One of the more surprising results of the anthocyanin stability study **[Chapter 2]** was the formation of monoacylated peonidins from diacylated peonidins under photo stress. Although the formation of new pigments have been reported in previous studies, to our knowledge no studies described this unique degradation pathway. [162-163] Due to the limitations of our study and this unforeseen result, our experimental conditions were not optimized to decipher the specific chemical mechanism involved. For instance, analytical conditions were not optimized to detect simple phenolic acids, primarily caffeic acid, which may have been a coproduct of monoacylated peonidins during the photo-degradation of diacylated peonidins from photo stress. If caffeic acid is in fact released intact, this may be a particularly interesting reaction for companies concerned with natural food colorants because intermolecular copigmentation may occur between these phenolics and result in a hyperchromic shift.[262-263] Another plausible mechanism may involve the oxidation of caffeoyl moiety prior to the cleavage from anthocyanins. Furthermore, several isomers of each acylated anthocyanin are possible, thus the location of the caffeoyl moiety is unknown. The position of the caffeoyl moiety may significantly contribute to the mechanism because of the protective effect induced by intramolecular copigmentation.[264] From our studies, these factors remain unknown and a new experiment must be designed to address these questions.

In order to answer these questions, an emphasis must be put on analytical procedures. High purity peonidin-3-dicaffeoylsophorosides-5-glucoside, -3-caffeoyl-*p*-hydroxybenzoylsophoroside-5-glucoside, and -3-caffeoylferuloylsophoroside-5-glucoside would either need to be purchased or isolated from purple sweet potato anthocyanin rich

extract. Initial structural characterization will need to be completed via two dimensional NMR. Once the positions of the acylated moieties are identified, di-acylated anthocyanins will be exposed to photo-stress at similar conditions as described in our previous study **[Chapter 2]**. Two different extraction and LC-MS methods are required for accurate quantitative and qualitative data of anthocyanins and other degradation products. For instance, our previous study utilized a solid phase extraction and LC-MS method that specifically targeted anthocyanins. Another method must be applied for caffeic acid and other potential low molecular weight degradation products. Finally, samples of the predominant compounds in each extraction would be structurally identified by NMR. From this information, the structural importance of acylated anthocyanins, the potential role of intramolecular copigmentation, and the mechanism of photo-degradation may be interpreted.

6.3 Flavonoids in Human Milk

The two studies on human milk flavonoid composition described in this dissertation provided significant insight to the direction of future research **[Chapters 3 and 4]**. High inter- and intra-individual variation was in part caused by the transient nature of flavonoids and variations in consumption. Thus, dietary questionnaires to approximate flavonoid consumption of lactating mothers may provide further insight into the composition of human milk. Furthermore, flavonoid plasma concentrations may not necessarily reflect milk concentrations because of their transient nature. However, urinary excretion data may be particularly useful to estimate overall bioavailability. Finally, a triple quadropole mass spectrometer may be able to detect and quantify

metabolites in single samples of milk, instead of pooled samples allowing for more accurate assessment of metabolite deposition in human milk.

6.4 Interactions Between Proteins and Flavan-3-ols

The study investigating the chemistry of flavan-3-ols in model dairy beverages concluded that the interactions between flavan-3-ols and dairy proteins drive a stabilizing effect for the flavan-3-ols **[Chapter 5]**. Although the study shed light on the interactions between flavan-3-ols and dairy proteins, questions concerning the mechanism of actions arose. A follow up study should consider a comparison with dairy protein isolates to target specific interactions. Furthermore, isolated EC, EGC, and ECG in these protein solutions should be investigated to avoid any potential competition between flavan-3-ols for binding sites. Additionally, THSN dimers may be generated and isolated, then exposed to dairy protein isolate solutions in order to elaborate on the potential interaction of auto-oxidation intermediates. Finally, LC-MS analysis may be optimized to detect flavan-3-ol and dimer quinones, both unbound and covalently bound to cysteine on enzymatic hydrolyzed peptides to provide further insight concerning these mechanisms.

One of the experiments in this study was designed to investigate the stability of flavan-3-ols in simulated human milk. However, this model may be improved to provide further insight concerning protein polyphenol interactions in human milk. The protein composition of human milk is different from bovine milk and may alter flavan-3-ol binding.[265] Furthermore, from our studies focusing on flavonoid concentration and composition in human milk **[Chapters 3 and 4]**, an accurate model should consider glucuronidated and methylated metabolites of various flavonoids. The various

conjugations are likely to impact the polyphenol protein interactions as they may disrupt the hydrophobic and hydrogen bonding forces that predominate.

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APPENDICES

Appendix A Acylated Anthocyanin and Flavonoid Metabolite Identification

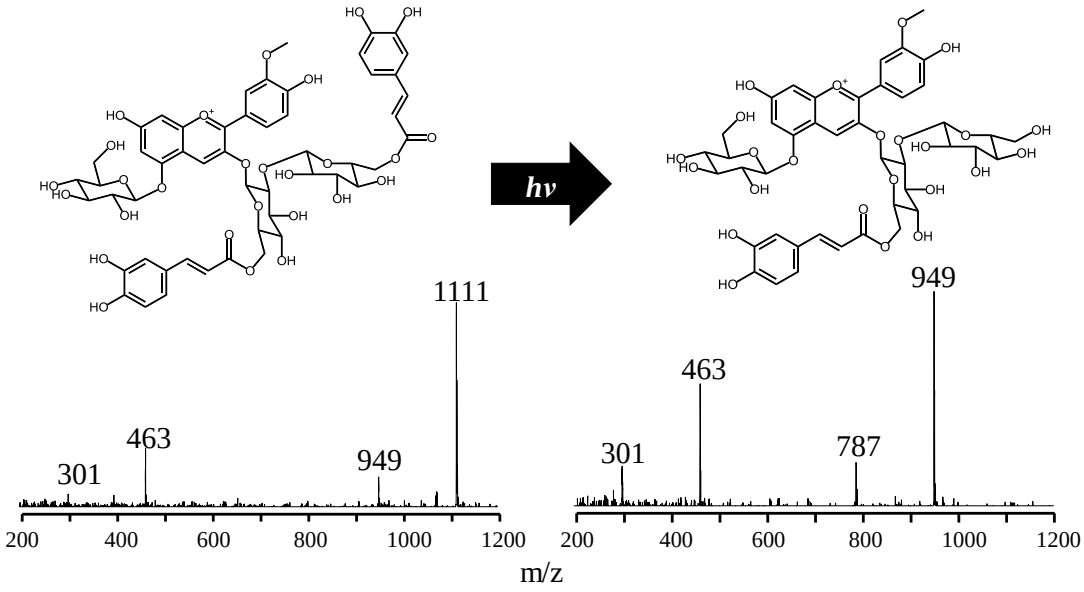

Figure A. 1 Structures and Mass Spectra of Peonidin-3-dicaffeoylsophoroside-5-glucoside and Peonidin-3-caffeoylsophoroside-3-glucoside Under Photo Stress.

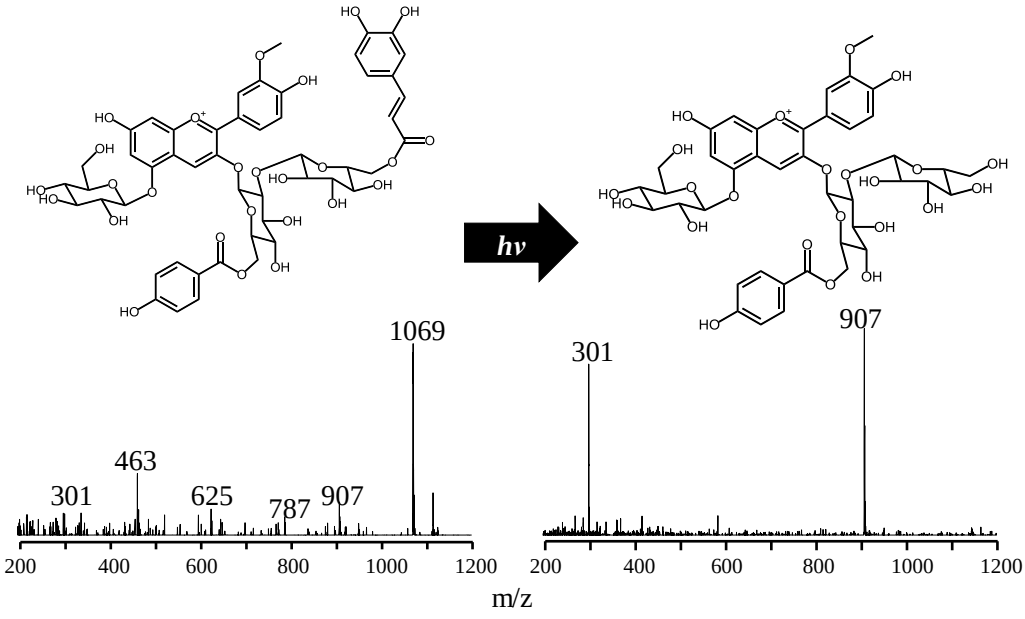

Figure A. 2 Structures and Mass Spectra of Peonidin-3-caffeoyl-p-hydroxybenzoylsophoroside-5-glucoside and Peonidin-3-p-hydroxybenzoylsophoroside-3-glucoside Under Photo Stress.

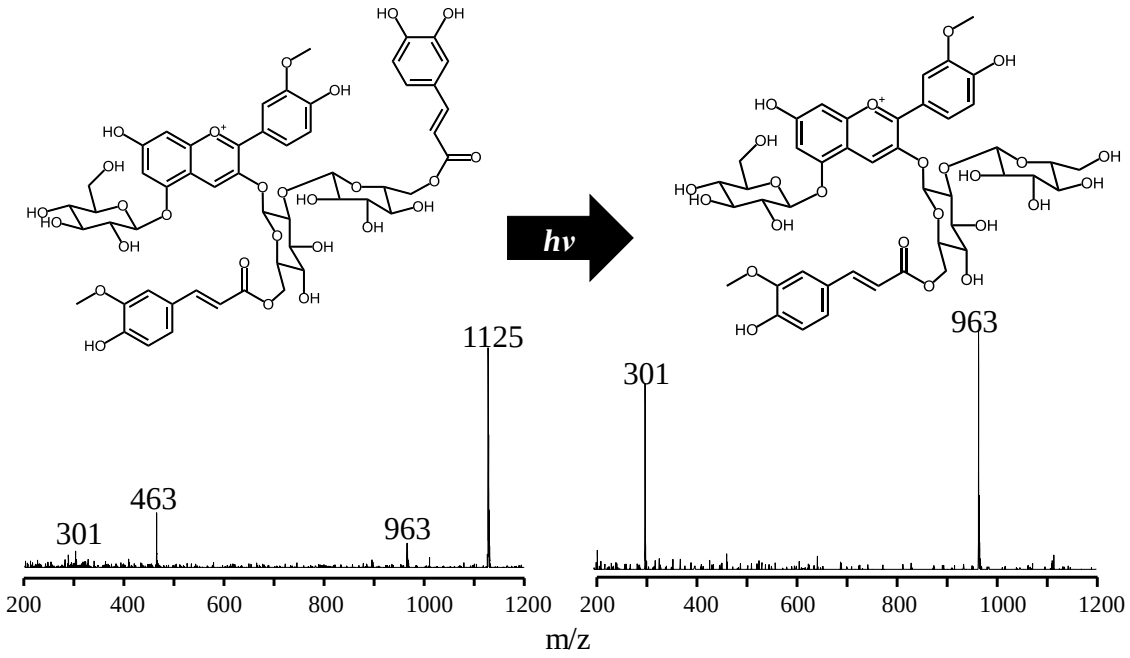

Figure A. 3 Structures and Mass Spectra of Peonidin-3-caffeoyl-feruloylsophoroside-5-glucoside and Peonidin-3-feruloylsophoroside-3-glucoside Under Photo Stress.

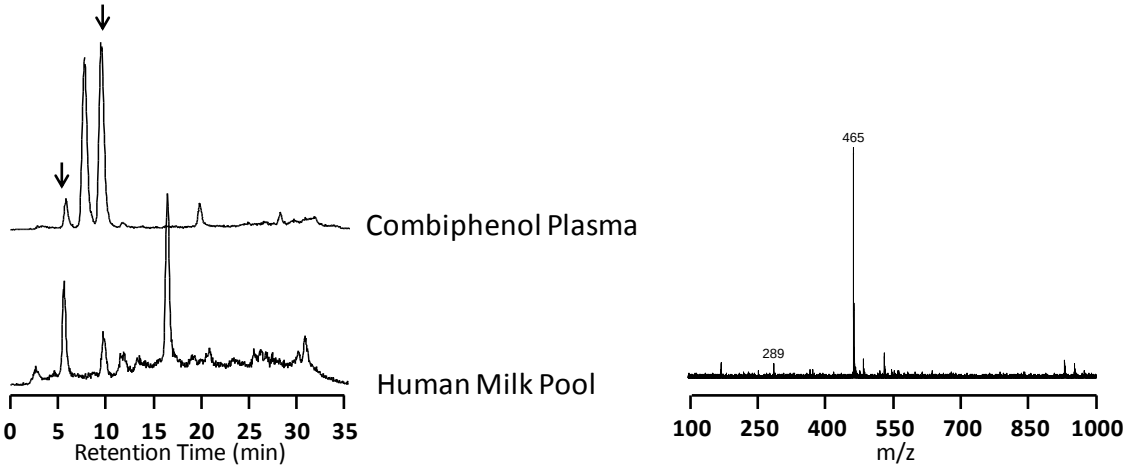

Figure A. 4 Chromatograms and Mass Spectra of Confirmed Epicatechin Glucuronides in Rodent Plasma and in Human Milk Pools.

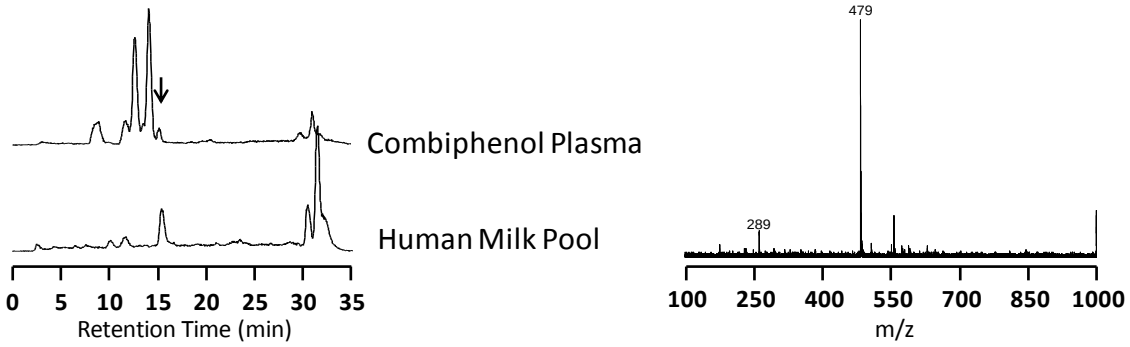

Figure A. 5 Chromatograms and Mass Spectra of Confirmed Epicatechin Methyl-Glucuronides in Rodent " Plasma and in Human Milk Pools.

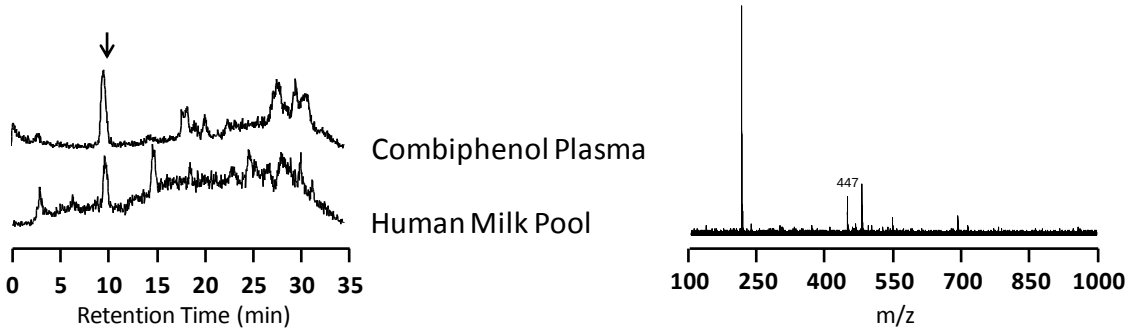

Figure A. 6 Chromatograms and Mass Spectra of Confirmed Naringenin Glucuronide in Rodent Plasma and in Human Milk Pools.

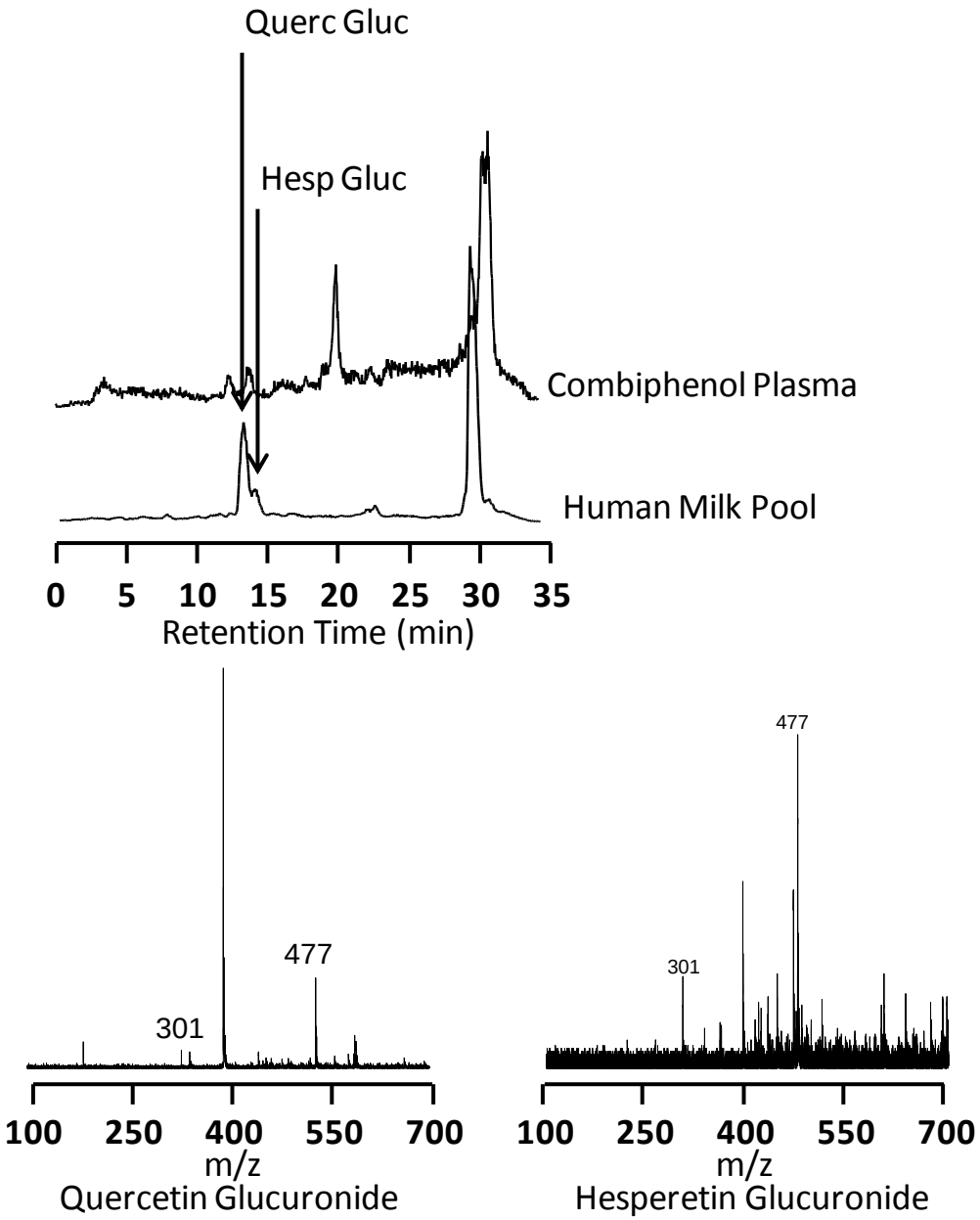

Figure A. 7 Chromatograms and Mass Spectra of Confirmed Quercetin and Hesperetin Glucuronides in Rodent Plasma and in Human Milk Pools.

Appendix B Research Methods

Solid Phase Extraction of Anthocyanins from Beverages

Objective: Purify and concentrate anthocyanins in a beverage.

Instrument: Solid phase extraction manifold
Rapidvap or Nitrogen manifold

Materials: Oasis HLB cartridge
DI water
Methanol
2% formic acid in water
2% formic acid in methanol
1 mL pipette
Disposable glass culture tubes

Procedure:

1. Obtain 1 Oasis cartridge for each sample and place onto manifold. Label cartridges to indicate sample.

2. Place a glass culture tube under each cartridge on the manifold.

3. Close the manifold, close the vent, turn on the vacuum.

4. Activate cartridge:
    a. Pipette 3 ml of methanol.
    b. Pipette 3ml of DI H_2O.

5. Pipette 4ml of each sample into their corresponding cartridge. Volume may be adjusted depending on the concentration desired. Note: if a colored solution is exiting the cartridge, it is saturated and a lower sample volume is required.

6. Pipette 2ml of formic acid in H_2O to rinse the cartridge. Turn off vacuum, open vent, remove manifold, and discard culture tubes

7. Label new culture tubes and place them under their matching cartridges. Close manifold, close vent, turn on vacuum

8. Pipette 2ml of formic acid in methanol into each cartridge. Remove culture tubes and evaporate methanol in either the Rapidvap or nitrogen manifold.

pH Differential Method to Determine Total Monomeric Anthocyanins

Objective: Determine total monomeric anthocyanins. Giusti et al. method modified to accommodate a 96 well plate reader.[7]

Instrument: Molecular Devices Spectra Max 100 with SOFTmax Pro software

Materials:
DI water
96 well plate
100 μL pipette
1 mL pipette
Multichannel pipette (Optional but highly recommended)
0.025 M potassium choloride buffer (pH=1.00)
0.400 M sodium acetate buffer (pH=4.50)

Procedure:

1. Turn on Molecular Devices Spectra Max 100 and open Softmax Pro. Allow 15 minutes for system to warm up.

2. Take 96 well plate and insert into drawer. Obtain a pre-reading of the plate.

3. Pipette 0.3 mL of DI water into a column. Note: You must program the template and designate water blanks, samples, and wavelength.

4. Pipette 60 μL of samples into two wells (A and B) per replicate. It is highly recommended that each is analyzed in triplicates (total of 6 wells per sample)

5. Pipette 0.24 mL of 0.025 M potassium choloride buffer (pH=1.00) in A wells and 0.400 M sodium acetate buffer (pH=4.50) in B wells.

6. Mix wells and wait 15 minutes.

7. Insert plate and read at 510nm and 700nm.

8. Calculate total monomeric anthocyanins by using this formula:
$[[(A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5}] * MW * DF * 1000]/ [\varepsilon * b]$

   Where A is absorbance at a given wavelength and pH, MW is the molecular weight, DF is the dilution factor (5 in this specific method), ε is the molar absorptivity, and b is the well height (1cm). Cyanidin-3-O-glucoside is typically used to estimate total monomeric anthocyanins (MW=449.2 g/mol and ε=26900 A/M)

Extraction of Flavonoids from Human Milk

Objective: Extract flavonoids for human milk and prepare samples for LC-MS analysis.

Instrument:
- Waterbath set to 37^{o}C
- Centrifuges
- pH meter
- Rapidvap or Nitrogen manifold

Materials:
- 4.07 x 10^{-6} g/L Ethyl Gallate Solution
- 2.74mM L-ascorbic acid/2.15 mM Na_2EDTA
- 40mg/ml pepsin solution in 0.1 N HCl
- 1926 kU/g B-glucaronidase
- Ethyl acetate
- 1.0 N NaOH
- Hexane
- 15 mL centrifuge tubes
- Glass culture tubes
- Glass pipettes
- Micro centrifuge vials

Procedure:

1. Obtain thawed human milk sample and transfer to 15 mL centrifuge tubes.
2. Transfer 1 mL of 4.07 x 10^{-6} g/L ethyl gallate solution (internal standard) and 50 µL of 2.74 mM L-ascorbic acid/2.15 mM Na_2EDTA to milk samples.
3. Create 40mg/ml pepsin solution in 0.1 N HCl and transfer 6 mL of solution to milk samples.
4. Place tube in shaking water bath for 15 minutes at 37^{o}C with gentle mixing.
5. Transfer 3 ml hexane, vortex solution, centrifuge, and discard hexane layer (top). Repeat once.
6. Titrate solution with 1.0 N NaOH until pH= 4.7.
7. Add 20 mg of 1926 kU/g B-glucaronidase to 10mL of deionized water. Transfer 1mL of enzyme solution to milk samples. Place in shaking water bath for 45 minutes at 37^{o}C.
8. Transfer 3 mL ethyl acetate, vortex solution, centrifuge, and transfer ethyl acetate layer (top) to glass culture tube. Repeat twice.

9. Evaporate off ethyl acetate by using the rapid evaporator or nitrogen manifold.

10. Dissolved dried pellet in desired ~200 µL mobile phase. Transfer into micro centrifuge vials and centrifuge on max RPM for 5 minutes.

11. Transfer into micro HPLC vials and analyze on LC-MS.

Appendix C First Order Degradation Rate to Degradation (%) Conversion Table

	First Order Degradation Rates (min^{-1})								
Time (min)	**1E-02**	**5E-03**	**1E-03**	**5E-04**	**1E-04**	**5E-05**	**1E-05**	**5E-06**	**1E-06**
0	100*	100	100	100	100	100	100	100	100
30	74	86	97	99	100	100	100	100	100
60	55	74	94	97	99	100	100	100	100
90	41	64	91	96	99	100	100	100	100
120	30	55	89	94	99	99	100	100	100
150	22	47	86	93	99	99	100	100	100
180	17	41	84	91	98	99	100	100	100
360	3	17	70	84	96	98	100	100	100
720	0	3	49	70	93	96	99	100	100
1440	0	0	24	49	87	93	99	99	100
2880	0	0	6	24	75	87	97	99	100
5760	0	0	0	6	56	75	94	97	99
11520	0	0	0	0	32	56	89	94	99
23040	0	0	0	0	10	32	79	89	98
46080	0	0	0	0	1	10	63	79	95
92160	0	0	0	0	0	1	40	63	91
184320	0	0	0	0	0	0	16	40	83
368640	0	0	0	0	0	0	3	16	69

*All values are presented as percents (%).

VITA

VITA

Brian J. Song

EDUCATION

Doctor of Philosophy West Lafayette, Indiana
Purdue University, Food Science **December 2013**

Bachelor of Arts New Brunswick, New Jersey
Rutgers University, Chemistry Major Food Science Minor **May 2009**

ACADEMIC EXPERIENCE

Purdue University, West Lafayette, IN
Doctoral Graduate Assistantship – Dr. Mario Ferruzzi 2009 - 2013

- Designed and executed experiments that investigated interactions between milk proteins and green tea flavan-3-ols.
- Investigated the degradation kinetics and formation of new pigments from anthocyanin rich extracts in model beverage systems through thermal and photo stresses by LC-MS analysis.
- Developed and tested the reproducibility and repeatability of analytical methods to detect polyphenols in human milk.
- Maintained, troubleshot, and repaired multiple LC-MS systems.
- Assisted in the analysis of polyphenols, degradation products, and their metabolites in diverse array of food, beverage, and tissue samples.

Rutgers University, New Brunswick, NJ
Undergraduate Research Assistant – Dr. Paul Takhistov 2008 - 2009

- Analyzed the physiochemical characteristics of hydrophobic pharmaceuticals in polymer matrixes via DSC and rheology techniques.
- Assisted in the development of an automated colloidal titrator for laboratory use.

Rutgers University, New Brunswick, NJ
Writing Center Tutor 2005 - 2006

- Instructed students in basic composition, expository writing, and scientific and technical writing.

INDUSTRIAL EXPERIENCE

Colgate-Palmolive Company, Piscataway, NJ
Intern / Co-Op Student **2006 - 2008**

- Developed, synthesized, purified, and analyzed novel organic polymers for oral pharmaceutical applications under the direction of Dr. Andrew Nowak.
- Analyzed the stability of flavors and texture of toothpaste samples at multiple conditions under the direction of Dr. Shira Pilch.

RESEARCH INTERESTS

Phytochemical Stability

- Reaction kinetics and mechanisms of phytochemical degradation
- Formation of novel degradation products with potential biological significance
- Impact of encapsulation
- Impact of food processing on phytochemical composition

Phytochemical Molecular Interactions

- Molecular interactions between polyphenols and macromolecules
- Intra- and intermolecular copigmentation of anthocyanins
- Impact on stability, degradation mechanisms, and bioavailability

Flavonoid Bioavailability

- Modulation of bioavailability through processing and food matrix interactions
- Investigation of the bioavailability of novel degradation products
- Delivery of flavonoids to targeted segments of the gastrointestinal tract
- Gastric transport of anthocyanins

PUBLICATIONS

1. Lipkie T.E; **Song B.J.**; Probst M.; Manganais C.; Jouni Z.E.; Ferruzzi M.G., Assessment of carotenoids and flavonoids in human milk in China, Mexico, and USA cohorts. (Currently in progress)
2. **Song B.J.**; Manganais C.; Ferruzzi M.G., The thermal stability of green tea flavan-3-ols in model dairy beverages. (Currently in internal review)
3. Wang, J.; Tang, C.; Ferruzzi, M. G.; Gong, B.; **Song, B. J**.; Janle, E. M.; Chen, T.-Y.; Cooper, B.; Varghese, M.; Cheng, A.; Freire, D.; Roman, J.; Nguyen, T.; Ho, L.; Talcott, S. T.; Simon, J. E.; Wu, Q.; G.M., P., Role of Standardized Grape Polyphenol Preparation as a novel treatment to improve synaptic plasticity through attenuation of features of metabolic syndrome. Molecular Nutrition and Food Research 2013, *In Press*.
4. **Song B.J.**; Sapper T.N.; Burtch C.E.; Brimmer K.; Goldschmidt M.; Ferruzzi, M.G., Photo and thermal degradation of anthocyanins from grape and purple

sweet potato in model beverage systems. (*Journal of Agricultural and Food Chemistry*, web published on January 18, 2013)

5. **Song B.J.**; Jouni Z.E.; Ferruzzi M.G., Assessment of phytochemical content in human milk during different stages of lactation. *Nutrition*. 29 (2013) 195-202
6. Neilson A.P.; **Song B.J.**; Sapper T.N.; Bomser J.A.; Ferruzzi M.G., Tea catechin auto-oxidation dimers are accumulated and retained by Caco-2 human intestinal cells. *Nutrition research (New York, N.Y.)* **2010,** *30* (5), 327-340.

PUBLISHED ABSTRACTS AND PRESENTATIONS

1. **Song B.J.**; Manganais C.; Ferruzzi M.G., The thermal stability of green tea catechins in model dairy beverages. Poster presentation at the International Food Technologists Conference. Chicago, United States. **2013**.
2. **Song, B.J.**; Brimmer K.; Goldschmidt M.; Ferruzzi, M.G., Kinetics and characterization of photodegradation products from purple sweet potato acylated anthocyanins. Poster presentation at the International Food Technologists Conference. Las Vegas, United States. **2012**.
3. **Song, B.J.**; Jouni, Z.E.; Ferruzzi, M.G., Assessment of phytochemical content in human milk during different stages of lactation. Poster presentation at the International Conference on Polyphenols and Health. Sitges, Spain. **2011**.
4. **Song, B.J.**; Sapper, T.N.; Hirt, S.A.; Brimmer K.; Goldschmidt M.; Ferruzzi, M.G., Photo and thermal degradation of anthocyanins from grape and purple sweet potato in model beverage systems. Oral and poster presentation at the International Food Technologists Conference. New Orleans, United States. **2011**.
5. **Song, B.J.** and Ferruzzi MG, Anthocyanin stability in model beverage systems. Oral presentation for Sensient Colors Inc. Saint Louis, MO. **2010.**

LEADERSHIP

College of Agriculture Advisory Committee of Graduate Students. 2011-Present

- Addressed the concerns of graduate students in the college of agriculture.
- Served as a liaison between food science graduate students and the college of agriculture.

USDA Civil Rights Compliance Review 2012

- Participated in a civil rights audit to ensure that graduate students were treated without bias due to race, gender, and ethnicity.

Food Science Graduate Committee Student Representative. 2011-2012

- Participated in discussions focused on the course work, requirements, and development of the food science graduate program.
- Served as a liaison between graduate students and faculty members.
- Represented the food science graduate program during the Whirlpool Engineering Rotational Leadership Development Program campus visit and Minorities in Agriculture, Natural Resources, and Related Sciences Region V Conference

TEACHING EXPERIENCE

Mentored several undergraduate researchers on various projects within Dr. Mario Ferruzzi's lab.

- Stacey Hirt **(2009 – 2010)**
- Claire Burtch **(2010 – Present)**
- Chris Manganais **(2012 – 2013)**
- Laura Willis **(2009 - 2010)**
- Michael Kohlmann **(2012)**

FS 453 Food Chemistry Lab Teaching Assistant **Spring 2013**

- Conducted pre-lab lectures to highlight concepts, experiments, and application of lab topics
- Supervised lab activities and encouraged detailed discussions of lab concepts

FS 467 Food Analysis **Spring 2011-2013**

Caffeine and Anthocyanin lab HPLC-PDA-MS demonstrations

- Lectured students on the theory, instrumentation, and application of LC-MS technologies
- Demonstrated sample preparation and analysis of beverage samples

FS 530 Food Ingredients **Spring 2012**

"Bioactive Compounds" guest lecture

AWARDS

International Food Technologists Conference **2012**

Food Chemistry Division Poster Competition: First Place

Purdue University, Ross Fellowship **2009-2010**

Rutgers University, Magna Cum Laude **2009**

The Croda Award **2005**

Outstanding Sophomore for Excellence in Organic Chemistry

PROFESSIONAL ORGANIZATIONS

International Food Technologists

Phi Tau Sigma Hoosier Chapter